MEDICINAL PLANTS OF NEPAL:

Ethnomedicine, Pharmacology, and Phytochemistry

by

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Abstract

Information about the medicinal uses of forty-two plant species was collected from traditional healers and knowledgeable villagers from a variety of different ethnic groups in Nepal. Illnesses for which these plants are used are those perceived in western style medicine to be caused by bacterial, fungal or viral pathogens. Methanol extracts of the species were screened for activity against a variety of bacteria, fungi and viruses, under various light conditions to test for photosensitizers. Thirty-seven extracts showed activity against bacteria and thirty-five showed activity against fungi. Only eight were active against Gram-negative bacteria. The exposure to UV-A light had a considerable effect on the activities of some extracts, with eight extracts being active only when exposed to light. The antibacterial and antifungal effects of fifteen extracts were enhanced upon exposure to light. Fifteen extracts showed 100% inactivation of at least one virus, and fifteen showed partial activity. Eight extracts were active only when exposed to light, and the antiviral effect of eight extracts was enhanced upon exposure to light.

A species showing antibacterial activity, *Centipeda minima* (Asteraceae), and one showing antiviral activity, *Carissa carandas* (Apocynaceae) were the focus of bioactivity guided fractionation. *Centipeda minima* was found to contain three sesquiterpene lactones, identified as 6-O-methylacrylylplenolin, 6-O-isobutyroylplenolin, and 6-O-angeloylplenolin. 6-O-Methylacrylylplenolin had not been previously isolated from *C. minima*. All three of these sesquiterpene lactones had activity against *Bacillus subtilis* and *Staphylococcus aureus*. A fraction from the methanol extract of *Carissa carandas* was quite active against herpes simplex virus. This fraction was found to contain a derivative of 3,4,5-trimethoxycinnamic acid.

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Forward

Portions of this thesis were previously written and published by the author. The full citations for these publications are as follows:

Taylor, R. S., Manandhar, N. P. and Towers, G. H. N. (1994). Activities of selected medicinal plants of Nepal. VIII International Conference, Traditional Medicine and Folklore, Newfoundland, Canada, In press.

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Taylor, R. S., Manandhar, N. P. and Towers, G. H. N. (1996). Antimicrobial and antiviral studies on selected medicinal plants of Nepal. Ethnobiology in Human Welfare: Fourth International Congress of Ethnobiology Ed. S. K. Jain. Lucknow, India, Shvam Arts, 79-82.

Taylor, R. S. L., Edel, F., Manandhar, N. P. and Towers, G. H. N. (1996). Antimicrobial activities of southern Nepalese medicinal plants. *Journal of Ethnopharmacology* 50, 97-102.

Taylor, R. S. L., Hudson, J. B. and Towers, G. H. N. (1995). Photo-mediated activities of antibacterial and antiviral compounds from plants. Phytochemicals and Health Eds. D. L. Gustine and H. E. Flores. American Society of Plant Physiologists, 30-40.

Taylor, R. S. L., Manandhar, N. P., Hudson, J. B. and Towers, G. H. N. (1996). Antiviral activities of Nepalese medicinal plants. *Journal of Ethnopharmacology* 52, 157-163.

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Plant material and ethnobotanical data were collected by the author, under the supervision of N. P. Manandhar. F. Edel participated in the extraction of the southern Nepalese plants, and their antibacterial and antifungal screening, under supervision of the author. Antibacterial and antifungal screening was done in the laboratory and under the supervision of

G. H. N. Towers, while antiviral testing was performed in the laboratory of J. B. Hudson, under his supervision.

....
R. S. L. Taylor

....
G. H. N. Towers

Chapter I
General Introduction

Ethnomedicine

All over the world, since ancient times, plants have been used for medicinal purposes. This knowledge has been passed down from generation to generation, the accumulation of which is evident in the wisdom of traditional healers. The age and depth of this tradition has been described by the world renowned ethnobotanist, Richard Evans Schultes, who claims that the traditional healer, "is usually an accomplished botanist, [and] represents probably the oldest professional man [sic.] in the evolution of human culture" (Lewington, 1990).

Evidence for the worship and medicinal use of sacred plants has been discovered dating back to the use of *Papaver somniferum*, the opium poppy, as long ago as 6,000 BC by Swiss Lake Dwellers (Lewington, 1990; Stockwell, 1989). The earliest systematic study of herbal medicine was made by the Chinese Emperor Shen Nung (ca. 2,700 BC). The Shen Nung Herbal (ca. 200 BC) mentioned the medicinal uses of 365 preparations, including the use of *Ephedra* species for bronchial problems, and *Ricinus communis* (source of caster oil) as a purgative (Mann, 1994).

Ethnobotanical information from all over the world, including herbal medicines which were a part of European culture, has led to the discovery of approximately 120 plant derived drugs, which account for about 25 percent of all drugs prescribed in North America every year (Cox and Balick, 1994). It

should also be noted that these 120 plant derived drugs do not include medicinal plants that act as bandages, glues, splints, and antiseptics, all of great importance to the medical community.

These 120 plant derived drugs surprisingly come from only 95 species of flowering plants. Less than 0.5% of all flowering plant species have ever been studied for potential pharmaceutical activity (Balick and Cox, 1996). With an estimated 265,000 species of flowering plants on the earth, it would seem that the search for medicines from plants has only just begun. Fortunately the scientific community, and society at large are now beginning to treat claims of the usefulness of medicinal plants seriously. This can be seen not only in the scientific literature of today, but also in the popular literature. For example, an article in Practical Gardening Magazine (James, 1992) not only describes the herbal riches to be found in the Chelsea Physic Garden, but goes on to explain that Glaxo, a leading pharmaceutical company, is testing many for biological activity.

A brief list of familiar drugs that are either made entirely from plants, derived from plants or are now made synthetically, but were first discovered in plants, includes the expectorant ipecac, the painkillers aspirin and morphine, the decongestant pseudoephedrine and the anti-cancer drugs vincristine and vinblastine. A more detailed list of commonly prescribed drugs from plants can be seen in Table 1.1.

It is crucial to keep searching for new medicinal compounds, not just to combat diseases for which there is no known cure, such as acquired immune deficiency syndrome (AIDS) and many forms of cancer, but to replace the antimicrobial drugs of today when pathogenic microbes acquire resistance to them. Antimicrobial compounds have made modern surgery possible; without them such procedures as open heart surgery, organ transplants and skin grafts

would not be feasible. Antibacterial and antifungal drugs are also needed to combat infection in immune suppressed patients. These patients often become infected with common microorganisms that healthy people could fight off. Antimicrobials are also often required to treat hospital acquired infections in people with a weakened immune system, such as newborns and the elderly.

Plants represent a vast pool of potential medicines, but as Cox and Balick (1994) point out, some collection strategy is needed because it is not feasible at this time to screen all the plants in the world for activity against all known diseases. Cox and Balick have classified the collection strategies into four groups. First there is the random search, which has not been very successful. The National Cancer Institute screened 114,000 plant extracts from 35,000 species for anti-cancer activity with no positive results. This type of screen is time consuming and expensive (Holland, 1994). Taxol©, the potent anti-cancer drug isolated from the Western Yew tree (*Taxus brevifolia* Nutt.) (Taxaceae) is a notable exception, as it was found in a random screen. Secondly, there is the phylogenetic survey, in which close relatives of plants known to produce beneficial compounds are selected. Ecological collections provide the third choice of survey, focusing on plants that display certain characteristics, which indicate that they are likely to produce chemicals that will have certain effects on humans. For example, plants that are immune to predation by insects most likely produce toxic chemicals that could be useful as medicinal agents.

Table 1.1

A partial list of commonly prescribed drugs that have been developed from plants, based on ethnobotanical information.

Medicinal use and plant source are also listed.

DRUG	MEDICINAL USE	PLANT SOURCE
Aspirin	Analgesic, anti-inflammatory	*Filipendula ulmaria* (Queen of the meadow) (now synthetic)
Atropine	Used in travel sickness medicine, to treat duodenal ulcers and used by ophthalmologists as pupil dilator.	*Atropa belladonna* - can now be synthesized, but starting point is dried leaves and flowering tops of *A. belladonna*
Contraceptive pill	Prevents pregnancy.	*Dioscorea* species (yams). Human hormones are synthesized from a steroidal sapogenin.
Digoxin, Digitoxin	Regulate heart beat while increasing strength and muscular activity of heart.	*Digitalis purpurea* (Foxglove)
Ephedrine Pseudoephedrine	Bronchodilator, stimulant and antispasmodic decongestant	*Ephedra* species
Hyoscine (Scopolamine)	Anticholinergic, used to treat motion sickness	*Hyoscyamus niger* (Henbane)
Ipecac	Induces vomiting. Alkaloids, especially ementine, are a specific cure for amoebic dysentery.	*Cephaelis ipecacuanha*
Morphine and Codeine	Powerful painkillers, codeine is also a cough suppressant.	*Papaver somniferum* Morphine cannot yet be synthesized.
Quinine	Anti-malarial.	*Cinchona pubescens*

Taxol©	Anti-cancer	*Taxus brevifolia*
Vinblastine Vincristine	Anti-cancer (vinblastine is used to treat Hodgkin's disease, vincristine to treat paediatric leukaemia).	*Catharanthus roseus* (Rose periwinkle)

Finally there is the ethnobotanical approach. Each culture has developed traditional herbal treatments based on their local flora. Unfortunately in developing countries, much of it is now being lost with the introduction of 'more scientific' Western health care, the destruction of the forests to feed and shelter the growing population, the exploitation of the land by foreign multinationals, and the loss of culture through assimilation by colonists, governments and missionaries. Cox (1994) explains that historically, ethnobotanical leads have resulted in three types of drug discovery. The first type is unmodified natural products where the ethnobotanical use suggested clinical efficacy. The heart drug digitalis fits into this category. The second type is the discovery of unmodified natural products where the ethnobotanical use only remotely suggested the clinical efficacy, such as the anti-cancer drug, vincristine. Finally, the third type is the greater efficacy of chemically modified natural or synthetic products that are based on the natural product used traditionally, such as aspirin.

Traditional herbal lore could prove to be invaluable to society, but must be documented before it is lost forever. This thought is summed up nicely by Schultes (1994):

> Ethnobotany is sometimes considered... a rather sentimental discipline, a form of scientifically sanctioned nostalgia for a simpler way of life. I would argue, however, that it is a highly practical discipline. The human species depends on plant species for its own welfare and ultimately its own survival. Each living species is the repository of organic molecules that are the products of the plants' irreproducible evolution. Science should intensify its study of these chemicals, because there is little time left to learn; when a plant becomes extinct, the opportunity to

learn is lost forever. It is only common sense that we who can apply technical analyses to problems should learn from peoples who are intimately familiar with their floral environment and its useful properties for the benefit of mankind [sic.].

Scope of Project

The aim of this research was to characterize pharmacologically active compounds from plants used medicinally in Nepal. This involved interviewing traditional healers in Nepal to obtain information on medicinal plants, collecting potentially active plants, screening them for biological activity, and then isolating and identifying the biologically active compounds. The project can be broken down into three distinct yet interconnected parts:

1) Ethnomedicine
2) Screening for pharmacological activity
3) Isolation and identification of bioactive constituents

The pharmacological activities assessed were antibacterial, antiviral and antifungal.

Infectious diseases are a great concern in Nepal, and as a result there are many herbal remedies used specifically to treat ailments caused by microorganisms. The symptoms of these pathogens are more easily identified than diseases such as 'cancer' or 'liver disease', thus facilitating a correct diagnosis. Tests for these activities had been previously established in the Towers and Hudson laboratories, so all necessary equipment and techniques were readily available.

It was hoped that not only would these medicinal plants provide interesting biologically active molecules, but also that by documenting this ethnobotanical information, both governmental and non-governmental agencies would have a valuable resource to use in discussions of reforestation, conservation of biological diversity, or rural health care.

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Chapter II

Ethnomedicine of Nepal

Introduction

Nepal (see Figure 2.1), a small country of 147,181 km^2 (official figure), nestled between India and Tibet in the Himalayan mountains, is about 800 km long, measured from northwest to southeast, and varies in width from 90 to 230 km. The land varyies in altitude from approximately 100 m above sea level in the plains of the Terai region in the south, to the highest point on earth, Sagarmatha (Mount Everest) at 8,848 m. This altitudinal range adds geographic diversity to the country, resulting in regions that vary from the vegatatively rich river valley jungles to the desolate mountain peaks of rock and scrub past the tree line.

Nepal is a poor country by Western standards, with a per capita income of the equivalent of $170 per year. Two-thirds of the country's income is supplied by foreign aid. The second and third sources of income are from tourism, and the wages of Gurkha solders paid mainly by Britain. The population of 18.5 million is expanding by a growth rate of half a million a year. Since 1991, masses of refugees from Bhutan (ethnic Nepalese) have been flooding Nepal and settling mainly on the overcrowded Terai region, adding to the over population problems (Clerc, 1993).

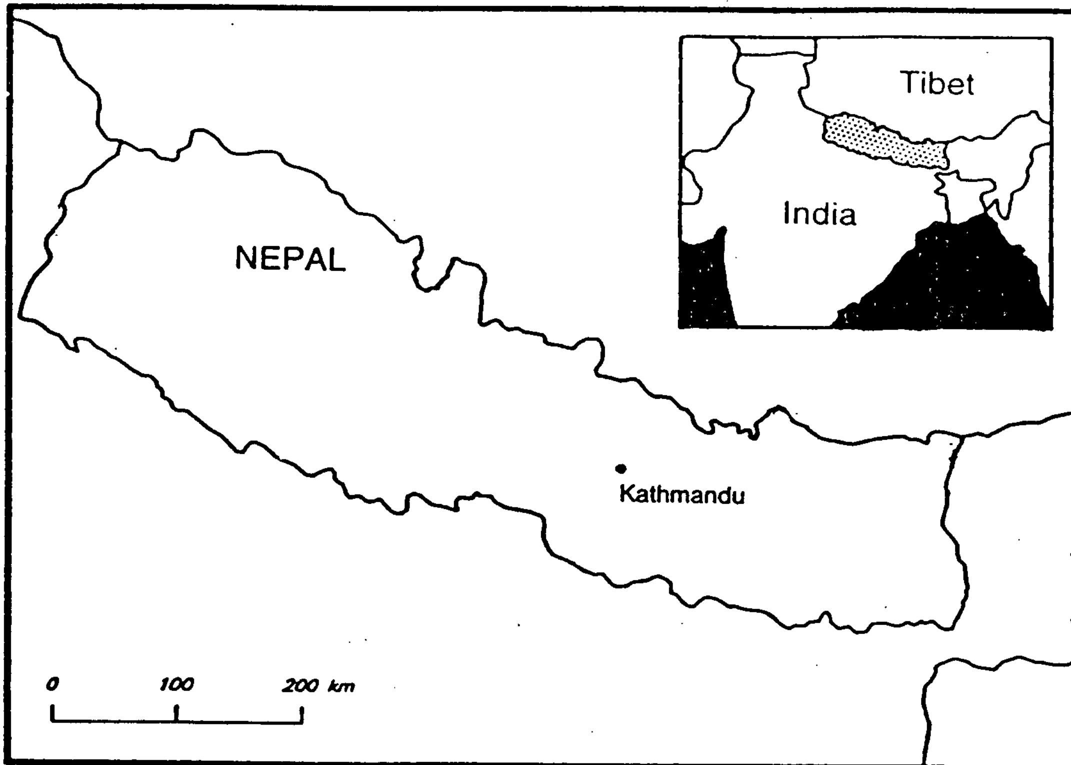


Figure 2.1. Map of Nepal

The usual problems of an overcrowded, developing country abound in Nepal. Poor sanitation problems lead to the spread of water-borne diseases, and simple health injuries such as cuts can easily develop infections and become life threatening. There are only 4,000 hospital beds in the entire country, and in 1987-88, the doctor patient ratio was 1: 20 000. The infant mortality rate, at 129 for every 1 000 live births, is four times that of China, and the average life expectancy is only about 50 years (Wheeler and Everist, 1990).

These problems are compounded by a literacy rate of less than 25% (Wheeler and Everist, 1990), and poor agriculture techniques, including very little sustainable farming or forestry. Deforestation not only leads to a lack of firewood and fodder, but also causes major landslides which happen each year during the monsoons. The ensuing great floods lead not only to massive destruction of lives, crops and homes, but also to the spread of water-borne diseases. They have been blamed for the frequent outbreaks of cholera, typhoid and gastroenteritis that follow flooding each year.

Diseases that are common in Nepal include cholera, meningitis, tetanus, diphtheria, typhoid and hepatitis. Water-borne pathogens include many bacteria, giardia, worms, amoebae and viruses. Insect bites or stings, cuts, and scratches that become infected, tuberculosis, rabies, and dengue fever are also important. To add to this, all the health problems that are experienced daily in the West, such as influenza, chicken pox, tonsillitis, coughs, and colds, are also present in Nepal, but inflict a much greater toll since malnourishment (and therefore weakened immune systems) is common, and unfortunately the treatment of rest and good food is not usually an option.

Nepal has long been recognized as having a wealth of medicinal plants. This is demonstrated in the Hindu myth where Lord Lakshman (Lord Ram's

brother) is injured in a battle, and Lord Hanuman, the Monkey God is sent to the Himalayas to find the only herb that will cure him. Hanuman reaches the Himalayas only to realize that he cannot remember which herb to collect, as there were many similar looking ones. To remedy this problem he collected a whole mountain, carrying it on his shoulder, and returned to the battle. The correct herb was then found, and Lord Lakshman was saved. Paintings of Lord Hanuman carrying the mountain (Figure 2.2) are very popular in Nepal, and one is even displayed as a wall mural in the major hospital in Kathmandu.

All these factors make Nepal an excellent site to conduct a study of plants used in traditional medicine. Over 90% of the population consists of subsistence farmers (Wheeler and Everist, 1990), most of whom have a deep connection to the land, and a vast knowledge of the local flora. As well, over 75% of the population is treated by traditional healers (Manandhar, 1980a). Nepal is very rural; in many instances the nearest city may be a six or seven day walk away. The use of traditional medicine continues because the country has many rural parts where Western pharmaceuticals or pre-packaged Indian Ayurvedic medicines are not available, and because these forms of treatment are much more expensive than herbal medicines. Furthermore, many families have traditional herbal treatments for minor illnesses (such as coughs, colds and sore throats) that do not require them to seek the advice of a traditional healer.

Those of the younger, more mobile generation in Nepal are familiar with the uses of the traditional medicine of their elders, but is happy to adopt the "easier" Western or Indian Ayurvedic medicines which can be taken straight out of the bottle or box, and which require no preparation time. For this reason it is necessary to record, preserve and promote ethnomedicinal knowledge so that it does not die out in the coming generations.



Figure 2.2. Lord Hanuman carrying a mountain

There are over thirty ethnic groups in Nepal, each with its own language, culture, religious beliefs and medical tradition. The rich ethnobotanical history of the area is documented in many studies conducted by Bhattarai (1992, 1993a,b,c), and Manandhar (1980b, 1985, 1986a, 1987, 1989a,b,c, 1990a,b, 1993, 1994, 1995a), but the actual medicinal values of the plants have not been ascertained in accordance with Western pharmaceutical practices. Collection of plant material is facilitated by the fact that walking is the accepted means of travel. The trails are for the most part safe, and some type of accommodation is usually to be found at the end of a day's walk. Since the country is so small, it is possible to collect plants in very different habitats within a reasonable time period.

Plant Selection and Collection

The plants in this study were collected under the guidance of Dr. N. P. Manandhar, of the National Herbarium and Plant Laboratories, Godawari, Lalitpur, Nepal. In each village, or in the fields along the trek, various native healers were questioned as to the medicinal uses of local plants. An effort was made to interview traditional healers, although the knowledge of villagers was also recorded. These people were easier to find, had more time to talk and who often offered unsolicited advice, was also recorded. The validity of this information was assured by checking it with at least five unrelated people, preferable from nearby villages, and also traditional healers. In order to facilitate recording of information, steps were taken to insure that the people being interviewed were comfortable with the procedure. This involved explaining the purpose of the collecting trip in the language of the villager, so

that informed consent could be given. The members of the collection team lived in the village with the villagers whenever possible, and tried to assimilate into the village life. This included eating and sharing meals with the villagers, swapping stories, living frugally and dressing appropriately.

The plants collected were selected according to the following criteria. They had to be used in traditional medicine to treat a disease or infection that is thought to be caused by a bacterial, viral or fungal pathogen. This included plants used to treat wounds, colds, coughs, diarrhoea, dysentery, venereal diseases, hepatitis and skin problems such as rashes, blemishes, boils and poxes. Only the part of the plant used in the traditional treatment was collected for analysis. Secondly, only plants that were common enough to provide a 500 g dried weight sample were collected.

Only those species consistently used to treat the same illness in several villages were selected. Two sets of voucher herbarium specimens was made for each collection, and these vouchers have been filed in the National Herbarium and Plant Laboratories, Godawari, Nepal, and in The University of British Columbia Herbarium (UBC).

Northern Nepal

Plant Selection and Collection

The areas travelled during the collecting trek included the Helambu and Langtang areas, north and northeast of Kathmandu, and also the Trisuli river valley area, to the west of Kathmandu (see Figure 2.3). Plants were collected over a period of three weeks, between May 20th and June 7th, 1993. This was the end of the 'summer season' and the beginning of the 'rainy season' in

Nepal, although the summer season had been unusually wet that year. The altitudes encountered on the trek varied from less than 600 m to approximately 4,500 m. Three distinct ethnic groups were encountered: Nerwaris, Tamangs and Sherpas.

Results

Twenty-one species from seventeen genera and thirteen families were collected. The species collected are listed in Table 2.1, with voucher specimen number indicated at the end of the corresponding ethnomedical field data.

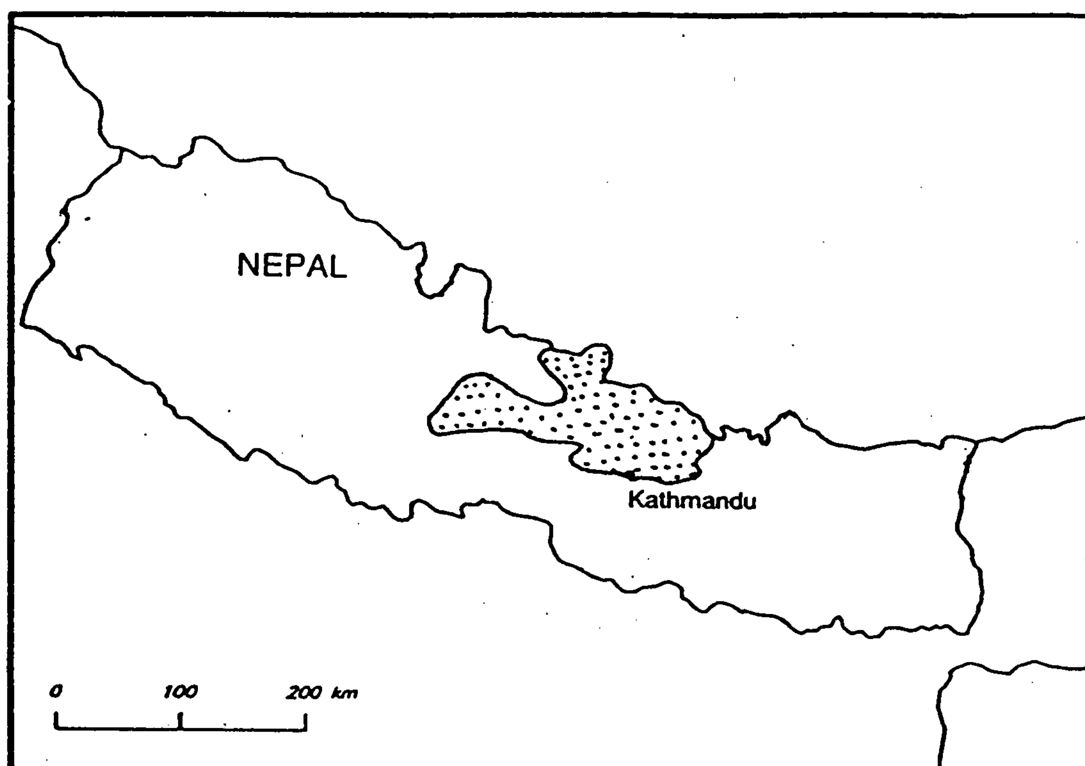


Figure 2.3. Collection Areas in Northern Nepal

Table 2.1

List of medicinal plants, their common names and medicinal use(s).

Voucher herbarium specimen is indicated at the end of field data,

followed by information from the literature.

Dosage information was difficult to quantify, but has been presented in 'teaspoon' or 'cup' measures, and should be taken as an approximation

ASTERACEAE

Centipeda minima (L.) A. Br. & Aschers

Nepali name: Hachhyun; Tamang name: Hachhi mran

Juice from the aerial parts of the plant is inhaled through the nose, and also four teaspoons of this juice are taken internally, three times a day, to treat coughs and colds (195-93 Q). The flower head is squeezed and the juice inhaled to treat headache and cough (Manandhar, 1993). Squeezed plant is inhaled to treat blocked nose resulting from coughs and colds (Manandhar, 1994).

Elephantopus scaber L.

Nepali name: Mulapati; Tamang name: Thagar

A paste made from the roots is applied to chicken pox lesions (17-93 E). Root powder is said to be an aphrodisiac (Manandhar, 1985). Nearly 5 g of root decoction is given to treat 'blood vomiting' (tuberculosis?) (Manandhar, 1987). The root is chewed to treat coughs and colds (Manandhar, 1989a). A decoction of the roots and leaves is given to treat diarrhoea and dysentery, while the plant paste is applied to cuts and wounds to prevent infection (Manandhar, 1989b).

Root juice, about four teaspoons, three times a day, is given for a week to treat fever and indigestion (Manandhar, 1995b).

CARYOPHYLLACEAE

Drymaria diandra Bl.

Nepali name: Abijalo; Tamang name: Abijal

Four teaspoons of plant juice are taken three times a day to treat coughs and colds (14-93 F). Plant paste or juice is applied to forehead to treat headache (Manandhar, 1987, 1989a). Two teaspoons of root juice are taken once a day (Manandhar, 1989a), or plant juice is given (two teaspoons, three times a day) (Manandhar, 1990b) to treat fever . The plant is used as a laxative. The paste is used to treat fevers caused by coughs and colds (Manandhar, 1989b). The plant juice is dropped in the eyes to treat conjunctivitis (Manandhar, 1989b) or eye disease (Shrestha and Joshi, 1993). For sinusitis, plant juice is inhaled from a cloth; plant juice, mixed with the juice of *Cheilanthes anceps* C. B. Cl (Pteridaceae) is taken (six teaspoons, twice a day) to treat peptic ulcers, as are four teaspoons of plant paste taken three times a day (Manandhar, 1994).

EUPHORBIACEAE

Macaranga pustulata King ex Hook f.

Nepali name: Malato; Tamang name: Kala

Bark juice is applied to treat skin blemishes (191-93 AA). The latex, applied around boils, is said to remove pus and quicken the healing process (Manandhar, 1994).

FUMARIACEAE

Corydalis longipes DC.

Nepali name: Pahenli

Three teaspoons of plant juice are taken three times a day to treat typhoid (8193 I).

GESNERIACEAE

Corallodiscus lanuginosus (Wall. ex DC.) Burtt

Nepali name: Kumkum

Six teaspoons of plant juice are taken twice daily to treat coughs and colds (185-93 X).

Didymocarpus primulifolius D. Don

Nepali name: Paharo ko kan

Two teaspoons of plant juice are taken four times daily to treat influenza (147-93 M).

HYPERICACEAE

Hypericum cordifolium Choisy

Tamang name: Marmhendo

The tender leaves are poisonous to animals (2-93 H). Plant juice (two teaspoons taken twice a day) is used to treat menstrual disorders, while root juice is taken for diarrhoea (Manandhar, 1990b). Flower paste (2 g) is eaten with warm water (three times a day for three days) to treat dysentery. Dried or preserved flowers may also be used (Bhattarai, 1993b).

H. elodeoides Choisy

Tamang name: Nachra mhendo

Root juice is used to treat fever (158-93 R). About 5 g of pounded root is given twice a day to treat fever (Manandhar, 1987).

H. uralum Buch.-Ham. ex D. Don

Tamang name: Nachra mhendo

Root juice is used to treat fever (64-93 B). Fresh or dried flowers (2-3 g) made into paste and taken with honey (three times a day for three to five days) for chronic dysentery (Bhattarai, 1993c).

LAMIACEAE

Elsholtzia blanda (Benth.) Benth.

Tamang name: lha silam

Plant juice and leaves are used to treat coughs and colds. The seeds are used to treat scabies (183-93BB). Four teaspoons of plant juice are taken four times a day for fever; leaf juice is applied on the back and chest of a child to treat coughs and colds (Manandhar, 1990c), while leaf juice is inhaled to clear nostrils congested from cough and colds (Manandhar, 1995b). Plant juice is also applied on the forehead and ingested (four teaspoons three times a day for two days) to treat headache (Manandhar, 1995b). Plants are tied around the waist to relieve backache (Shrestha and Joshi, 1993).

E. flava (Benth.) Benth.

Nepali name: Ban silam

Five grams of root are taken three times a day to treat fever (87-93 J).

E. fruticosa (D. Don) Rehder

Nepali name: Sano silam; Tamang name: Mranja

Five grams of root are taken three times a day to treat fever (145-93 L).

Micromeria biflora (Buch.-Ham. ex D. Don) Benth.

Nepali name: Pinase dhar; Tamang name: Masinomran

Two teaspoons of plant decoction are given twice a day for one week to treat coughs and colds (19-93 A). Two to four drops of plant juice are inhaled through each nostril two to three times a day for a week or longer to treat sinusitis (Bhattarai, 1993a). The plant is rubbed and inhaled to stop nose bleeds (Manandhar, 1987). Plant juice (one teaspoon) is ingested and/or inhaled to treat sinusitis (Manandhar, 1989a). Pieces of the root are pressed between the jaws to treat toothache (Manandhar, 1995a).

Pogostemon benghalensis (Brum. f.) O. Kuntze (18-93 C)

Tamang name: Rasangan

To treat coughs and colds, four teaspoons of plant juice are taken four times a day for four to six days (18-93 C). Plant juice is mixed with year old cow dung and given to cattle to control dysentery (Manandhar, 1985, 1989c,d, 1990a). About two teaspoons of root juice are given two times a day to treat coughs and colds (Manandhar, 1989a). Root juice is given to relieve fever (Manandhar, 1989b). Four teaspoons of root juice are taken twice a day for indigestion (Manandhar, 1990c). Root (Manandhar, 1993) or plant juice (Manandhar, 1995b) is added to water to bathe feverish children. Plant juice may be applied to the forehead during this procedure (Manandhar, 1995b).

LYGODIACEAE

Lygodium japonicum (Thunb.) Sar.

Tamang name: Janai langdu

The aerial parts of the plant are made into a paste which is applied to treat herpes (1-93 D). A plant paste is applied to treat joint ache (Manandhar, 1989a). Plant juice is applied to treat herpes (Manandhar, 1990b).

MYRSINACEAE

Maesa macrophylla (Wall.) A. DC.

Nepali name: Bhogati; Tamang name: Bhogati

The bark is boiled and the extract gargled to treat tonsillitis (155-93 N). The leaf juice is used as a fish poison (Manandhar, 1987). Fruit juice is applied to treat scabies (Manandhar, 1989a). Fruit juice is given to animals to treat diphtheria (Manandhar, 1989c). Fruit juice is given to animals to treat haemorrhagic septicaemia (Manandhar, 1990a). Leaves are roasted in an earthen pot, powdered and applied to boils (Manandhar, 1990b).

RANUNCULACEAE

Anemone obtusiloba D. Don

Nepali name: Ratanjot; Tamang name: Rikabe

Three teaspoons of root juice are taken three times a day to treat coughs and colds (171-93 Y).

RHAMNACEAE

Zizyphus mauritiana Lam.

Nepali name: Bayar; Tamang name: Bayar

Ring worm is treated by an application of root paste (194-93 P). Wood of this plant is used in both sacrificial fires and for funeral pyres. Ripe fruits are eaten. A root decoction is used to treat fever, while the powdered roots are applied to old wounds and ulcers. Bark is taken to treat diarrhoea (Majupuria and Joshi, 1989). The ripe fruits are eaten as food (Manandhar, 1982). Fifty grams of powdered plant gall is given twice a day to treat dysentery (Manandhar, 1985). Two teaspoons of root juice is given twice a day to relieve excessive menses (Manandhar, 1987). Two teaspoons of root juice are given once daily to treat stomach disorders (Manandhar, 1989a). Leaves are used to treat bleeding gums, and also liver troubles, diarrhoea and conjunctivitis. Bark juice is given to treat diarrhoea and dysentery. The unripe fruits are said to cause coughs and increase thirst, while the riper fruits are regarded as being good to treat fever, ulcers and wounds, to purify the blood and to ease digestion. Root decoction is used to treat fever. Dried branches are used as fencing (Manandhar, 1989b). The paste of the ripe fruit is given to treat fever (Manandhar, 1990a). Seed paste is applied to treat measles (Manandhar, 1994).

ROSACEAE

Princepia utilis Royle

Ethnobotanical information was taken from literature. This species was collected by N. P. M. in September 1993.

Warm seed oil is smeared on chest and throat at bed time to treat coughs and colds (Bhattarai, 1993a). Warm seed oil is massaged into chest to relieve

chest pain (Bhattarai, 1993c). The seed oil is used for cooking and as lamp fuel (Manandhar, 1982). The seed oil is applied externally for rheumatism, and pains resulting from fatigue. A heated oil cake is applied externally to treat stomach aches. In the Jumla district of Nepal the plant is used as a soap for washing clothes. The oil is used for cooking and in lamps when no other is available. The shrub is also used to make fences (Manandhar, 1989b). Paste of the oil cake is applied to treat ring worm or eczema. Warm seed oil is applied to treat body ache, especial after hard physical labour (Manandhar, 1995a). Fruit paste is applied to warts (Shrestha and Joshi, 1993).

Sibbaldia micropetala (D. Don) Hand.-Mazz.

Nepali name: Bhui pasari jhar

Six teaspoons of plant juice are taken four times a day to treat diarrhoea and dysentery (141-93 K).

VALERIANACEAE

Valeriana jatamansii Jones

Tamang name: Nakali jatamasi

Root juice used to treat eye infections and pimples (159-93 Z). The root is used to treat epilepsy. Root paste is used to treat headache and eye trouble (Manandhar, 1980a, 1986a). A plant paste is applied on boils (Manandhar, 1987).

Discussion of the Northern Nepalese Collection

From the twenty-one plant species collected, there are sixteen that are used to treat illnesses that are potentially viral in nature, sixteen to treat illnesses that are probably caused by bacteria, three for possible fungal diseases, and one that is used to treat an ailment most likely caused by insects. Most frequently the whole plant is used in these treatments, especially when for small, herbaceous species. When only the root is used, it is usually from a perennial, woody species. In only three of the treatments are soley aerial parts used. In two cases it is bark, and in one, seeds that are used.

The most common way of taking the treatment is to ingest the plant material. Thirteen out of the twenty-one plants are used this way. The two methods of preparation encountered were decoction, or extraction by boiling, and ingestion of the freshly squeezed plant juice. Six of the treatments are for illnesses seen to be external, such as skin blemishes, eye infections, ringworm, chicken pox and herpes lesions. These illnesses are treated with an application of either fresh plant juice or paste to the affected area. Only one treatment involves inhaling plant juice.

The two families with the most species collected were Lamiaceae with five species and Hypericaceae with three species. This does not give a valid representation of frequency of family use because an active attempt was made to collect *Hypericum* and *Elsholtzia* . It was known from previous investigations that several *Elsholtzia* species (Yip, 1993) and the compound hypericin, from *Hypericum perforatum* (Hudson et al., 1993b), have antiviral activity.

The Nepalese medicinal usage of seven of the plant species collected had not been previously documented. One species was *Corydalis longipes* of the Fumariaceae, which is used to treat typhoid, a bacterial infection caused by *Salmonella typhi*, resulting in fever, headache and diarrhoea. Two members of

the Gesneriaceae family, *Corallodiscus lanuginosus* and *Didymocarpus primulifolius,* are used to treat coughs and colds and influenza, respectively.

The two species of *Elsholtzia*, *E. flava* and *E. fruticosa*, are both taken to treat fever. *Anemone obtusiloba* (Ranunculaceae), is used to treat coughs and colds, and the seventh previously unrecorded Nepalese medicinal plant, *Sibbaldia micropetala* (Rosaceae) is used to treat diarrhoea and dysentery.

Southern Nepal

Plant Selection and Collection

The plants were collected in the Dhading District of Central Nepal, and the Banke, Kanchenpur and Dandeldhura Districts in Southwestern Nepal (see Figure 2.4) from December 12, 1994 to January 1, 1995. They were selected because of their use among the Tharu, Magar, Chhetri, Newaris and/or Raute people to treat illnesses possibly caused by bacterial, fungal or viral pathogens. They included plants used to treat cuts, wounds, colds, coughs, diarrhoea, dysentery, fever, sore throats, tonsillitis, and skin problems such as rashes, blemishes, boils and poxes. Only those species which were consistently used to treat the same illness in several villages were selected. The information was gathered by questioning local healers and knowledgeable villagers.

Healers and villagers who were especially helpful, and who were among those who provided the ethnobotanical information in this study, were Suraj Prasad Chaudhary (plant numbers 378-14, 382-17, 381-16, 108-20), Lok Bahadur Dhami (259-12), Suntali Dhami (247-11), Ram Bahadur Thapa Magar (29-5, 60-21), Suntali Magar (17-4), Renuka Mishra (10-1), Radhika Pandey

(145-7), Kamalsing Raute (379-15, 262-13), Gopal Shreshthe (11-2, 14-3), Jogi Ram Tharu (384-19), Kushi Ram Tharu (242-10).

Two sets of voucher herbarium specimens were made for each collection, and these vouchers have been filed in the National Herbarium and Plant Laboratories, Godawari, Nepal, and in The University of British Columbia Herbarium.

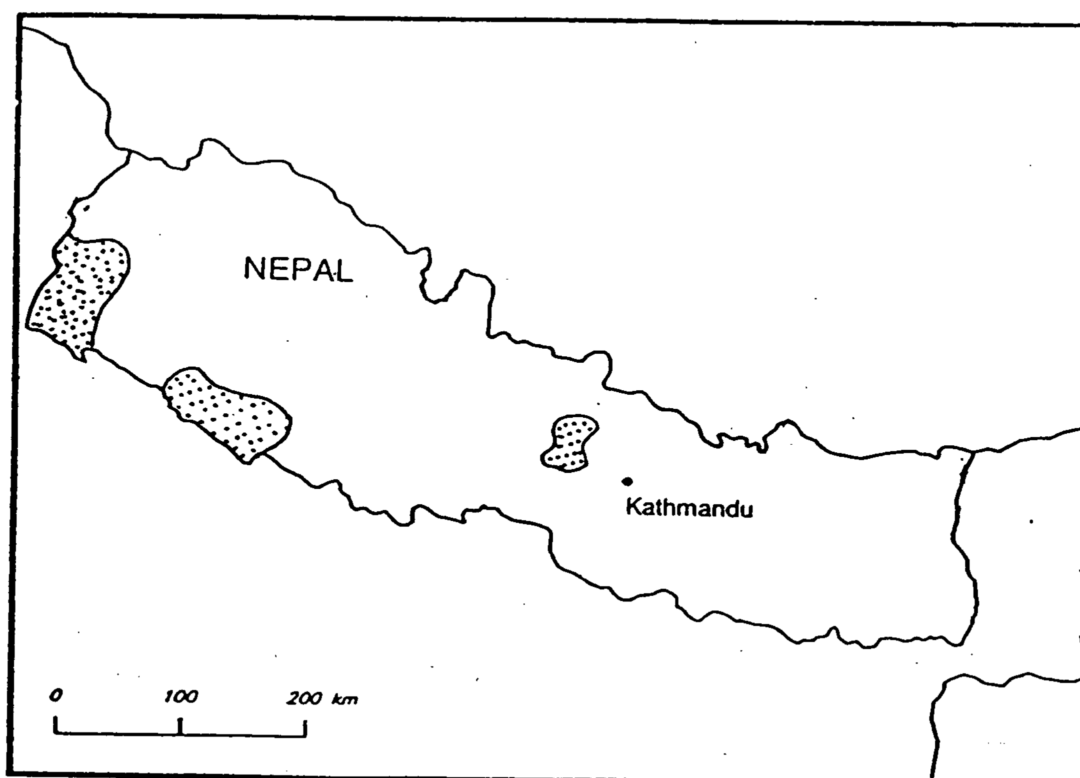


Figure 2.4. Collection Sites in Southern Nepal

Results

Twenty species from thirteen families were collected. They are listed in Table 2.2, with voucher specimen number indicated at the end of the corresponding ethnomedical field data. A photo of traditional healer Gopal Shreshthe treating a small female child for 'side pains' has been included (Figure 2.5). Gopal Shreshthe is a renown traditional healer, with patients visiting from an area having a radius of up to two days walk. The brother of the child being treated had also been treated by Gopal Shreshthe for the same ailment when he was younger. Gopal Shreshthe first examined the child and questioned the mother about the pain, then prepared a small vessel of water in which he placed various herbs. He then dipped a flower into the water and splashed the child with drops while chanting various mantras. After this he 'cleansed' the mother by wetting her hair with the same water and also chanting mantras while he ran his hands over her head. Once this procedure was finished, the traditional healer and the mother of the sick child made an offering of rice and turmeric powder to the gods, and the healer was paid one 'manna' (measure) of rice.

Gopal Shreshthe said that this was the first stage of the healing, and that he would sleep that night, and in his dreams the gods would tell him which medicinal plants to use to treat the child the following day.

Table 2.2

List of medicinal plants, their common names and medicinal use(s).

Voucher herbarium specimen is indicated at the end of field data,

followed by information from the literature.

Dosage information was difficult to quantify, but has been presented in 'teaspoon' or 'cup' measures, and should be taken as an approximation

ACANTHACEAE

Peristrophe bicalyculata (Retz.) Nees

Tharu name: Chuchure

Plant juice is used to treat fever, while a paste of the plant is used to treat cuts and wounds (190-8).

Rungia parviflora (Retz.) Nees

Raute name: Aankhyala

Plant juice is taken for fever. Dose is six spoonfuls three times a day (379-15). Plant paste is applied on cuts and wounds (Manandhar, 1990b).

AMARANTHACEAE

Alternanthera sessilis

Nepali name: Aankhle; Tharu name: Saranchi

Fresh plant juice is taken for fever. One half to three quarters of a cup taken three times a day. Also aerial parts of plant are boiled and taken as a tea for coughs and colds (378-14). The tender portions are eaten as vegetables (Manandhar, 1982). The plant is mixed with the buds of *Ficus lacor* L. (Moraceae) and the leaves of *Dalbergia sissoo* Roxb. ex DC (Fabaceae) and

the pounded mixture is added to water prepared as a bath to treat heat stroke (Manandhar, 1985). Plant juice is applied to treat cuts and wounds (Manandhar, 1986b). Plant juice (two to three teaspoons) is taken twice daily to treat white discharge in urine. The juice is also used to treat scabies (Manandhar, 1989a). A paste of the plant is used to treat venereal diseases and is applied to wounds to accelerate healing (Manandhar, 1989b). Root juice (three teaspoons, two times a day) is given to treat dysentery (Manandhar, 1990a). Root juice (two teaspoons, three times a day) is given for fever and dysuria. Plant paste is mixed with corn flour, baked and eaten for menstrual disorders (Manandhar, 1990b).

APOCYNACEAE

Carissa carandas L.

Tharu name: Karondath

Root juice is used to treat diarrhoea and dysentery. A dose of 1/2 to 3/4 of a cup is taken twice a day (382-17). Ripe fruits are eaten as food and also pickled (Manandhar, 1982). Nearly 2 g of root paste is given to control dysentery of cattle. Root powder is said to eliminate lice (Manandhar, 1985). Decoction of young leaves is given to treat intermittent fever (Manandhar, 1986b). The ripe fruits are eaten to treat diarrhoea, while the unripe fruits are made into preserves. The thorny branches make excellent fences, and are also burnt as fuel (Manandhar, 1989b). Root decoction is given to treat dysentery in cattle (Manandhar, 1989d). Root juice dropped inside wound (animal) to remove worms or germs and helps to accelerate healing (Manandhar, 1995a).

The Tharu people living in the Bahraich District of Uttar Pradesh, India (on the Indo-Nepalese border), use the fruits of *C. carandas* to treat diarrhoea

in animals. A water decoction (100 mL) of the fruits is given only once (Singh et al., 1996).

ARACEAE

Scindapsus officinalis (Roxb.) Schott

Nepali name: Hata kalla

Used for diarrhoea and dysentery in animals (cattle and goats). The fruit is cut in slices and given with food (247-11). Fruit is softened by heating and then fed to animals to treat stomach (intestinal?) worms (Manandhar, 1993).

ASTERACEAE

Eupatorium odoratum L.

Nepali name: Ban masuwa. The Nepali name means 'Forest Destroy', because when the forest has been destroyed it grows in great abundance.

Juice of the aerial parts is applied to cuts and wounds. It is said to be as good as iodine for preventing infection and halting blood flow (11-2). Leaf juice is applied to cuts and wounds (Manandhar, 1989a, 1990a, 1995b).

Inula cappa (Buch.-Ham ex D. Don) DC.

Magar name: Gai tihare

Root juice (three spoonfuls, three times a day) is taken to treat fever (29-5). A paste made of root powder is used to treat fever (Manandhar, 1980b, 1990c), as is a root decoction (two teaspoons twice a day) (Manandhar, 1990b). Bark juice, mixed with the bark of *Myrica esculenta* Buch.-Ham. ex D. Don (Myricaceae) and *Ficus semicordata* Buch.-Ham. ex Sm. (Moraceae) in equal parts, is useful for menstrual disorders (two teaspoons a day). Washing with a decoction of the roots is recommended for body ache (Manandhar, 1990b).

Root juice (two teaspoons, three times a day) is given for indigestion (Manandhar, 1990c, 1995b), and for peptic ulcers (four teaspoons, three times a day)(Manandhar, 1993).

Tridax procumbens L.

Nepali name: Kurkure

Plant juice is applied to cuts and wounds (17-4). The flower head is chewed to treat coughs and colds. Plant paste is applied to boils (Manandhar, 1985). Plant juice (two teaspoons, two times a day) is used to treat fever (Manandhar, 1989a). The whole plant is mixed with animal feed to treat haemorragic septicaemia of animals (Manandhar, 1990a). Plant juice is dropped into the eye to treat cataracts (Manandhar, 1993).

COMBRETACEAE

Terminalia alata Heyne ex Roth

Nepali, Tharu and Raute name: Saj

Fresh bark juice is taken for diarrhoea and dysentery. Three spoonfuls taken three times a day for as long as necessary (262-13). Bark juice is applied to cuts and wounds (Manandhar, 1989a, 1990c, 1995b). Resin is applied to treat swelling caused by injury (in humans and animals) (Manandhar, 1993).

EUPHORBIACEAE

Mallotus philippensis (Lam.) Muell.-Arg.

Nepali name: Sịndure. (The plant is named for the red seed powder used to colour the traditional part in the hair of married women.)

Bark juice is taken for diarrhoea and dysentery. Three spoonfuls taken three times a day for as long as necessary (144-6). Crushed fruits (2-3 g)

without seeds are eaten with molasses (two times a day for two to five days) to expel tape worms. The red powder (obtained from mature fruits) mixed with butter (cow's), is applied to skin infections like ringworm and scabies (Bhattarai, 1993c). Bark juice is used to treat diarrhoea (Manandhar, 1989a). The red powder covering the ripe fruit is said to kill intestinal worms, and is also used as a purgative. A paste from the leaves is applied to treat wounds (Manandhar, 1989b). Bark juice is taken (two teaspoons, twice a day) for stomach disorders (Manandhar, 1990b).

The Tharu people living in the Bahraich District of Uttar Pradesh, India (on the Indo-Nepalese border), use the glandular hairs of the fruits mixed with coconut oil and apply this as a light massage to skin affected by scabies. This treatment is done at least three times a day for a month. They also use a powder of the dried seeds (10 g), mixed with a little salt to treat stomach ache. This remedy is taken with water. To kill lice, powdered fruits (5 g) are taken orally with water, once daily for three to five days. (Singh et al., 1996).

Jatropha gossypifolia L.

Nepali name: Sajyon

Four spoonfuls of bark juice are taken three times a day for coughs and colds (145-7). Twigs are used to treat athlete's foot and wounds of the feet (Manandhar, 1986b). Plant latex is applied to treat toothache (Manandhar, 1986b, 1990a).

FABACEAE

Bauhinia vahlii Wight & Arnott

Nepali name: Bhorla

Root juice is applied to cuts and wounds (241-9). Seed paste is applied to suppress boils in their early stages (Bhattarai, 1993c). Roasted seeds are eaten as food (Manandhar, 1982). The mature seed is pounded and applied to boils, especially those of children (Manandhar, 1985). The seeds are said to have aphrodisiac properties, and are considered a tonic. The bark is made into a rough rope, and the leaves are used as plates for food and formed into cups for drinking water while working in the fields (Manandhar, 1989b). Root is pounded and then eaten with yogurt to treat dysentery (Manandhar, 1990a). Root juice (four teaspoons, twice a day) is used in amoebic dysentery (Manandhar, 1993).

Milettia extensa (Bentham) Baker

Nepali name: Gaujo

Juice is applied to treat infected wounds, and to treat scabies. The area is soaked, then scratched (to allow juice to penetrate) and then juice is applied. Both root and aerial parts of plant are used, although root juice is said to be more effective (60-21{root} and 16-21b {aerial parts}). Plant juice is mixed with water and used as a wash to rid cattle of ticks (Manandhar, 1987). Root juice is mixed with water and used as a body wash to remove lice or other parasitic insects from an animal (Manandhar, 1989d).

MALVACEAE

Sida cordata (Brum. f.) Borss.

Nepali name: Bishkhapre

Root paste is applied on cuts, boils and wounds (10-1). Root decoction is given as a tonic (Manandhar, 1985). Root paste is mixed with copper sulphate and butter (made from cow's milk) and is applied to treat gonorrhoea and other venereal diseases (Manandhar, 1986b). It is also applied to take the pus out of boils and wounds (Manandhar, 1990b). Root juice is applied to treat cuts and wounds (Manandhar, 1990a). Leaf juice is applied to treat pimples and boils (Manandhar, 1993).

Urena lobata L

Nepali name: Kuro

Seed powder taken after a meal with hot water to treat diarrhoea and dysentery. Four teaspoonfuls are taken twice a day for as long as necessary (14-3). Fruit paste is given to treat diarrhoea (Manandhar, 1986b). Leaf juice is applied to boils to remove pus and accelerate healing (Manandhar, 1995a). Plant juice, mixed with the juice of *Cissampelos pareira* L. (Menispermaceae), is taken to treat stomach disorders (Manandhar, 1990b), while root juice (six teaspoons, four times a day for six days) is taken orally to treat indigestion (Manandhar, 1995b).

MORACEAE

Streblus asper Loureiro

Tharu name: Sehor

Bark juice is taken (three to four teaspoons, twice a day) with cold or warm water to treat diarrhoea and dysentery. The water is used to make the

juice easier to swallow, as it does not taste good. The bark juice is extracted by pounding the bark into a paste, placing the paste into a clean piece of cheesecloth, and wringing it to obtain the juice (381-16).

POLYGONACEAE

Rumex hastatus D. Don

Nepali names: Amile, Chukya

Root juice is taken as needed for tonsillitis and sore throat. The root is also chewed and a paste applied externally (259-12). The tender shoots and/or leaves are pickled or mixed with other vegetables for the sour taste (Manandhar, 1982) . Root juice (two teaspoons, three times a day) is given to treat diarrhoea and dysentery in children (Manandhar, 1995a). The root is cleaned and chewed to treat sore throats (Manandhar, 1993).

RUTACEAE

Aegle marmelos (L.) Correa.

Nepali, Tharu and Raute name: Bel

Root juice pressed and taken for fever. The dose is four teaspoonfuls, three times a day (242-10). Pulp of ripe fruit is mixed with water and given to cattle orally to treat burns (Bhattarai, 1992). To cure dysentery, about 25 g of ripe fruit pulp are eaten twice a day for three to five days. Also, the stem bark is combined in a 2:1 mixture with the flowers of *Hypericum cordifolium* and a spoonful of this is taken three times a day for two to three days for diarrhoea. This preparation can be dried for storage (Bhattarai, 1993b). Bruised leaves (5 g) boiled in water (300-400 mL) and strained, then drunk warm with honey two to three times a day for three or more days is used as an antipyretic drug (Bhattarai, 1993c). The wood of this tree is never burned as it is considered to

be sacred. The fruits are often worshipped in Hindu ceremonies. The root is used to treat intermittent fevers and also is used as a fish poison. The fruits are used to treat chronic diarrhoea and dysentery. The fresh juice of the leaves is given with honey to treat fever and catarrh, and is also dropped in the eyes to treat eye problems. A hot poultice of leaves is applied to the forehead to treat fevers, and to the chest to treat bronchitis. Decoction of the root bark is given to treat heart palpitations, and intermittent fevers (Majupuria and Joshi, 1989). The unripe fruits are used to cure diarrhoea, while the ripe fruits are eaten to treat constipation, and also made into a sweet drink. Poultices of the leaves are used to treat eye diseases (Manandhar, 1980a). Pulp of the ripe fruits is eaten (Manandhar, 1982). Paste of unripe fruit pulp is used to treat diarrhoea (Manandhar, 1986b). Fruit pulp is given in cases of diarrhoea (Manandhar, 1989a). Leaf juice is applied to forehead to treat headache. Root is used to treat fever. Pulp of ripe fruit is taken to treat bowel complaints (Manandhar, 1990c). Wood ash is smeared on swollen body parts, especially when accompanied by fever (Manandhar, 1993).

VERBENACEAE

Clerodendrum indicum (L.) Kuntze.

Tharu name: Chuchure

To treat fever, two spoonfuls of fresh plant juice (aerial parts) are taken three times a day. Plant paste is applied to cuts and wounds (108-20). Root juice (four teaspoons, once a day) is given to treat diarrhoea (Manandhar, 1990c). To treat malnutrition, root juice is given (four teaspoons a day) on Sunday and Tuesday for one month (Manandhar, 1995b).

Lippia nodiflora (L.) Riche.

Nepali name: Ganthe

Plant juice (4-6 teaspoons, three times a day after meals) is taken for fever. Foods containing a lot of oil and/or chili are avoided (384-19). Plant is immersed in water overnight and the next morning half a teaspoon of the paste is taken to treat headache (Manandhar, 1985). Plant juice (two teaspoons) is taken to treat fever (Manandhar, 1989a, 1990c). Squeezed plant is inhaled to treat cough and cold (Manandhar, 1990c, 1995b).



Figure 2.5

Gopal Shreshthe treating a young girl for 'side pains'.

Discussion of the Southern Nepalese Collection

Out of the twenty plant species collected, there are twenty-five treatments that are used in southern Nepal to treat illnesses that are most likely caused by bacterial infection. Eighteen species are used to treat possible viral infections, one species is possibly insecticidal, and only one species is used for possible fungal ailments. The part of the plant most frequently used in these treatments is the root, followed by the whole plant or the aerial parts. Usually the whole plant is used when it is small and herbaceous, while aerial parts are used when the plant is larger, or has woody roots. In four cases bark is used, and in only one of the twenty-five treatments are seeds or fruit used.

Approximately two thirds of the herbal treatments are taken internally, with ingestion of fresh juice being the most prescribed treatment. Preparation of a tea, or chewing/eating pieces of the plant or the preparation of a powder of the plant which is eaten as, or with, food are also used. External applications involve either fresh juice or a paste.

The plant material used is usually fresh, possibly because fresh plant material is available on the Terai all year long. The Tharu people who were interviewed said that they preferred fresh plant material, but they would also use dried material when fresh was not available. The Rautes of the Jogbuda area, lead by Chief Kamalsing Raute, said that they would rather use fresh material. If the most effective plant is not available as fresh material, a second species may be used to treat the illness. In these cases a higher dosage would be given. If this second choice is not available, then a third species may be used, and an even higher dosage would be given.

The information given by Kamalsing Raute was recorded, and the Raute tribe was then revisited two days later to confirm the information, and also to discuss non-medicinal ethnobotanical practices. When Kamalsing Raute was

shown *Terminalia alata* and asked for the medicinal usage, he laughed and replied that the medicinal usage was still the same as it was two days ago, and that it would still be the same in a month, a year or even ten years.

Alternanthera sessilis (Amaranthaceae) was documented as being used by Suraj Prasad Chaudhary to treat fever, which is likely to indicate a viral, bacterial, or parasite infection. Other ethnobotanical uses in the literature indicate that it is also used to treat cuts and wounds (Manandhar, 1986b), white discharge in urine (Manandhar, 1989a), dysentery (Manandhar, 1990a), fever, and dysuria (Manandhar, 1990b). All these illnesses could be caused by bacterial or viral pathogens. The common usage among different peoples in different areas of Nepal gives credibility to the claim that *Alternanthera sessilis* has medicinal value.

General Discussion

The term 'ethnobotany' was coined by John W. Harshberger in 1895 to describe the study of "plants used by primitive and aboriginal people." The 1896 publication of The Purposes of Ethnobotany is now taken as the starting point for ethnobotany as an academic discipline. One hundred years have passed, and the term ethnobotany has come to describe a discipline composed of a variety of fields. Balick and Cox (1996) describe ethnobotany as "the study of relationships between plants and people." It is a "field of study that analyzes the results of indigenous manipulations of plant materials together with the cultural context in which the plants are used." Indigenous peoples are described as "peoples following traditional non-industrial lifestyles in areas they have occupied for generations." Schultes and von Reis (1995) define

ethnobotany as "the study of human evaluation and manipulation of plant materials, substances, and phenomena, including relevant concepts, in primitive or unlettered societies."

With these interdisciplinary definitions of the field it is easy to understand Balick and Cox's (1996) concept of an ideal ethnobotanist as a combination of an "anthropologist, archaeologist, botanist, chemist, psychologist, ecologist, explorer, folklorist, pharmacologist and diplomat." They insist that it is necessary to "see plants through the healer's eyes", a thought that is mirrored by Prance (Prance, 1994), who says that when dealing with other cultures, one must stop thinking of disease in Western terms and try to understand the concepts and spiritual side of the medicine of the people with whom one is working.

The choice of plants used to treat infectious diseases was appropriate for this investigation for many reasons. Infectious diseases are rampant in Nepal. Villagers and families as well as traditional healers are familiar with their treatment. The infrastructure to test the plant activity against microorganisms (bacteria, fungi and viruses) *in vitro* was present in the Towers and Hudson laboratories at The University of British Columbia, making the screening process manageable. The final reason for choosing to collect plants used to treat infectious diseases was the relative ease of explaining the symptoms of microbial infection to the villagers and healers. 'Khoki', 'rugha', and 'kateko ra ghau, (colds, cough, and cuts and wounds) are all words known to the average Nepali, just as they are to the average Canadian. The source of illness causing the symptoms associated with a microbial infection is difficult to determine. A red and puffy area around a cut that is oozing pus is most likely to be a bacterial infection, but a fever may be a result of any type of microbial infection. The microbes responsible for a case of diarrhoea or dysentery are also difficult to

diagnose without culturing them in a lab. The Nepalese healers make distinctions between diarrhoea (usually translated as diarrhoea and dysentery), which could be cause by viral or bacterial infection, and bloody diarrhoea, (what is medically termed dysentery) which, in Nepalese results, is usually caused by amoebae. Other diseases such as malaria, typhoid, and cholera have such typical symptoms that they can usually be correctly identified by the healers.

In this study, plants were chosen if they were used to treat a symptom that is usually associated with a microbial infection. They were grouped into categories based on the most likely source of illness. For example, plants used to treat cuts were categorized as potential antibacterials. Plants used to treat fever were categorized as both potential antibacterials and potential antivirals, although it is recognized that the sole effect of the plant may be to reduce body temperature, which, if the case, would eliminate it from the antimicrobial category. This chance was seen as unavoidable as there was no way of distinguishing between the two.

The mode of preparation, dosage and any other instructions involving the medicine were all recorded with no attempt to judge the validity of the claim based on western medical ideas. The only criterion the information had to meet was that it was confirmed by at least four other healers or respected villagers in the surrounding area.

The evidence for using ethnobotanical leads for medicinal investigations is convincing. MacRae et al. (1988) determined that approximately 80% of medicinal members of the Amazonian Euphorbiaceae were biologically active. Cox and colleagues (Balick and Cox, 1996) had similar results, with 86% of medicinal plant species in Samoa to show significant levels of pharmaceutical activity. Balick (Balick and Cox, 1996) found that a group of 'powerful plants'

from a healer in Belize gave four times as many "hits" in an HIV screen than a random sampling of plants.

Plants are not always considered 'medicinal' by the society in which they are used, even if they have physiological properties that would be considered medicinal in the west. An example of this is the diet of the Masai people of Kenya and Tanzania, which consists primarily of meat, milk and blood. The Masai have a surprisingly low blood cholesterol level. Timothy Johns and his colleagues (Johns, 1990) have studied the plants that are added to the meat and milk dishes, and have determined that they do reduce blood cholesterol. Are these plants food, or medicine?

In Nepal, several of the medicinal plants collected are also used as food, sometimes as delicacies and sometimes as a source of food when the regular choices are scarce. For example, the root of *Elsholtzia blanda* is taken to treat fever by the Sherpa people of the Langtang region, but the seeds have also been used by the Sherpa people of Darjeeling District, India (many Nepalis travel freely across the border) as a substitute for 'dal' or pulse (Yonzone et al., 1996). Some plants that are used medicinally are also used as food spices and seasonings, such as garlic and ginger.

The reasons for documenting the traditional usage of plants spread far beyond the search for new drugs from plants. The information can be used by both government agencies and non-governmental organizations (NGO) to combat such problems as deforestation and loss of biodiversity. In Nepal, there are NGO projects being set up to reforest areas with trees that have little value to the locals, such as *Eucalyptus*. With no knowledge of the plants, and no use for them, the villagers do not care for them and the trees die (Manandhar, 1995c). The rural people of Nepal have a very close relationship to the earth, and every plant has not only a name, but a use. Even children know if a plant is

edible, poisonous, medicinal, good for fodder or good for firewood. If reforestation efforts concentrated on trees that are useful to the community there would be possibly a greater chance for their survival.

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Chapter III
Antimicrobial Screening

Introduction

Microbes are responsible for a large percentage of disease, not just in Nepal, but all over the world. In Nepal, infections occur mainly from unsanitary conditions typical of a developing country, for example unclean drinking water, overcrowding, poverty and poor nutrition. In the West these conditions are not as prevalent, but microbial infections are still common. Besides the usual array of viral infections such as coughs and colds, and the communicable childhood diseases such as measles and chicken pox, hospital-acquired, or nosocomial infections are a concern both in the West and in developing countries. The Center for Disease Control estimates that 5 - 15% of all hospitalized patients in the United States develop a nosocomial infection, with 20,000 dying each year (Tortora et al., 1989). The most common nosocomial infections are urinary tract infections from urinary catheterization. Other common infections are surgical wound, lower respiratory, and cutaneous infections (Neu, 1993). The increasing use of antibiotics has led to drug-resistant strains of bacteria and fungi which in turn has increased the incidence of nosocomial infections. Two of the microorganisms used in this study, methicilin-resistant *Staphylococcus aureus* and multi-resistant *Pseudomonas aeruginosa*, are major problems in hospitals, with resistance to all antibiotic agents. *Staphylococcus aureus* causes pneumonia, bacteraemia and infects surgical wounds, while *Pseudomonas aeruginosa* is responsible for urinary tract infections,

bacteraemia ans pneumonia (Neu, 1993). A list of these and other common hospital pathogens can be seen in Table 3.1.

There have been many antimicrobial compounds isolated from plants. Bérdy's (1982) handbook of antibiotics from higher plants gives a good overview, as does Grayer and Harborne's (1994) review of antifungal compounds. Plants possess a great array of antimicrobial compounds, varying in structure from terpenoids (including monoterpenes, sesquiterpenes, diterpenes, terpene lactones, steroid alkaloids and steroids), to aromatic and aliphatic compounds, to carbohydrates, lactams, quinones and peptides. From studies conducted on medicinal plants of British Columbia in the Towers laboratory in the past few years, the following antimicrobials have been isolated and identified: chlorochimaphilin, a chlorinated naphthoquinone from *Moneses uniflora* A. Gray (Ericaceae) (Saxena et al., 1996), a thiophene (7, 10-epithio-7,9-tridecadiene-3,5,11-triyne-1,2-diol, from *Balsamorhiza sagittata* (Pursh) Nuttall (Asteraceae) (Matsuura et al., 1996b), and several bibenzyls from *Empetrum nigrum* L. (Empetraceae) (Matsuura et al., 1995).

The bacterial and fungal strains used in this screening were selected for their relevance as either hospital pathogens (e.g. Methicilin resistant *Staphylococcus aureus*, *Serratia marcescens* and *Candida albicans*), or as a model of an important pathogenic microorganism. As many organisms as possible were tested, in order to develop a full antimicrobial profile of the Nepalese extracts. More information about the diseases caused by the bacteria in trhis screening can be obtained from Isenberg and Painter (1978), Ivler (1978), and Hugh and Gilardi (1978).

Table 3.1

A list of common hospital-acquired (nosocomial) infections

Bacteria	Resulting Infection
Enterobacteria - *E. coli, Klebsiella* species, *Enterobacter* species, and *Serratia marcescens*	Urinary tract infections, peritonitis, bacteraemia, pneumonial infections, septicaemia, gastrointestinal inflammations, and neonatal meningitis
Staphylococcus aureus	Urinary and respiratory tract infections, endocarditis (inflammation of the lining of the heart)
Enterococci	Urinary tract and surgical wound infections
Pseudomonas aeruginosa	Burn and surgical wound infections, septicaemia and pneumonia

Different microorganisms are affected by antibiotics, and antimicrobial compounds found in plant extracts, in different ways. An example of this can be seen in the effectiveness of penicillin. Penicillin prevents the linking of the 3-dimensional peptidoglycan cell wall in Gram-positive bacteria, resulting in eventual cell rupture and death. Gram-negative bacteria are not seriously affected by penicillin.

Antimicrobial drugs are the class of therapeutic agents used to treat bacterial diseases by interfering with the growth of organisms. Antibacterial compounds can work in one of two ways, being either bactericidal (killing the organism) or bacteriostatic (inhibiting growth of the microorganism). If the compound is bacteriostatic, the host's immune system usually destroys the invading microbes. Tortora et al. (1989) list the following as criteria for evaluating antimicrobial drugs: the drug should be selectively toxic against the microorganism without harming the host; it should not cause an allergic reaction in the host; it must be have a half life long enough to remain in the infected tissue and destroy the pathogen before it is metabolized and excreted from the body; and it should be stable at refrigerator temperatures for extended periods of time.

Many crude plant or fungal extracts show enhanced antimicrobial activity when tests are carried out in the presence of light. Often there is no "dark activity". It is thus surprising that papers (Vlietinck et al., 1995) and even reviews (Grayer and Harborne, 1994) continue to be published in which this light factor is completely ignored. The activity that is light-dependent or light-enhanced is caused by the presence in the extracts of natural photosensitizers, compounds which, in their excited state brought about by light, react with other molecules in the test cell or organism, generally resulting in the death of the cell.

The influence and potential effects of light should always be considered in the search for new antimicrobials from plants. In this research, if a crude extract tests positive for antimicrobial or antiviral activity in the dark, it is referred to as being "dark active". The experiment is always replicated with a treatment in which the test organism is incubated with the extract in the presence of UV-A radiation and/or visible light. If there is enhanced activity or activity which was not indicated in the dark treatment, this is ascribed to the presence of a photosensitizer acting as an antimicrobial agent in the extract. Many species of plants have light-mediated activity because of the presence of natural photosensitizers. This topic has been covered extensively in reviews by Hudson (Hudson and Towers, 1991; Hudson, 1995), Taylor et al. (1995b) and Towers (1980, 1984; Towers and Hudson, 1987). This consideration becomes important in drug discovery, because clearly there is little reason to re-isolate well-known photosensitizers with antibiotic activities (e.g. furanocoumarins), when investigating the Rutaceae, Apiaceae, Moraceae, and other families which are characterized chemically by the presence of these compounds.

Natural photosensitizers of plants include quinones, porphyrins, polyynes, phenylpropanoid derivatives and aromatic alkaloids. The wavelengths of light needed to photoactivate these compounds vary from near UV to red, depending on the chromophores of the photosensitizers. Ultraviolet-A (UV-A) (320-400 nm) and visible radiation, unlike the shorter wavelengths of UV-B (290-320 nm) and UV-C (200-290 nm) do not cause direct damage to cells or tissues unless compounds absorbing at these wavelengths are accumulated in them. Plants that produce and accumulate these potentially toxic chemicals usually sequester them in glands or laticifers. For example, hypericin, an extended anthraquinone of *Hypericum* species, is stored in leaf glands while the thiarubrines, sulphur heterocyclic derivatives of polyynes, are

sequestered in cortical canals which run parallel to the longitudinal axis of the root or stem (e.g. in *Chaenactis douglasii*).

Different species of microorganisms may also react differently to extracts or pure compounds when exposed to them under different light conditions. For example, thiarubrine A is active against the yeast *Candida albicans* in the dark and under UV-A radiation, while it is not active against Gram-negative *Escherichia coli*. However, it is active against Gram-positive *Staphylococcus aureus* only in the presence of UV-A radiation (Ellis, 1993). This phenomenon is seen in Figure 3.1.

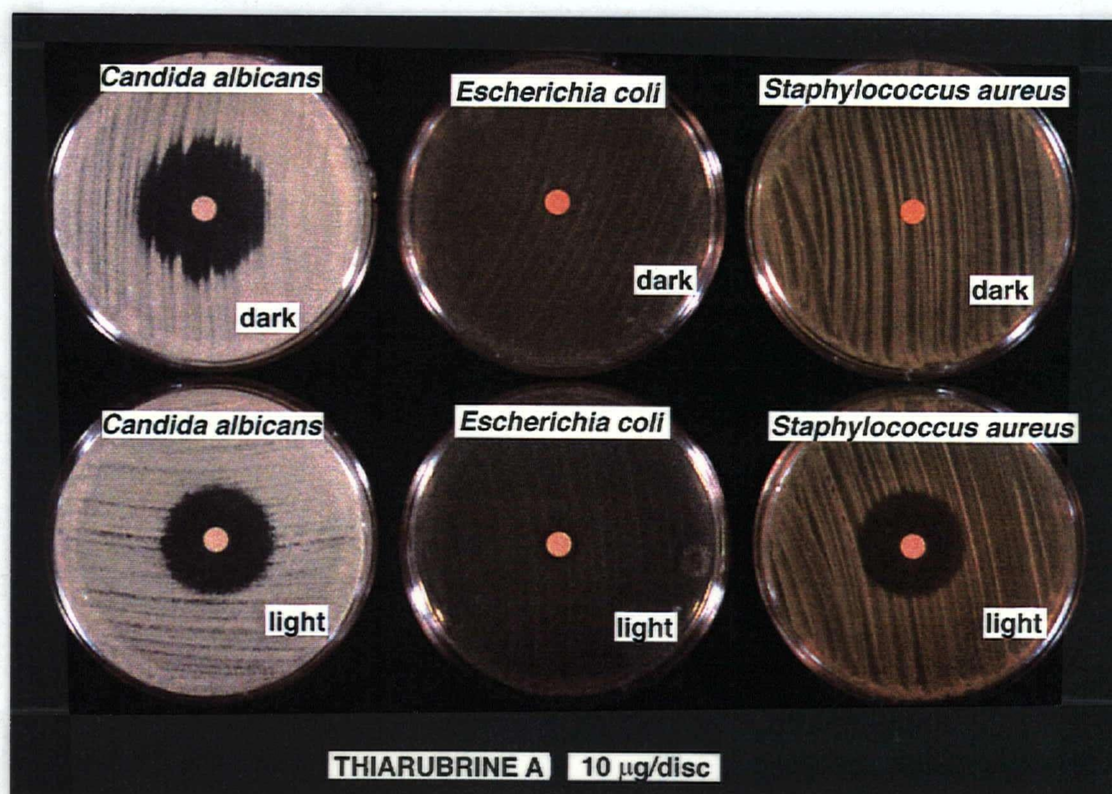


Figure 3.1. Disc assay of thiarubrine A against a variety of microorganisms in light and dark

Antimicrobial Screening of Northern Nepalese Medicinal Plants

Methodology

Extract and Disc Preparation

Plant material was air dried and ground in a Wiley grinder with a 2 mm wire mesh. A 20 g sample of the powder was soaked in 500 mL methanol (MeOH) for a minimum of 24 h. The sample was then suction filtered through Whatman # 1 filter paper, and washed with another 500 mL MeOH. The filtrate was evaporated to near dryness under reduced pressure and redissolved in 10 mL MeOH. This gave an extract of 2 g of dried plant per mL. Paper discs were impregnated with 20 µL of extract, corresponding to 40 mg of dried plant material, and allowed to dry at room temperature.

Microorganisms

Eight species of bacteria and five species of fungi were used in the screening process. The Gram-positive bacterial strains were *Bacillus subtilis, Staphylococcus aureus,* and *Streptococcus faecalis.* The Gram-negative *Escherichia coli, Serratia marcescens, Salmonella typhimurium,* and *Pseudomonas aeruginosa*, and the acid fast *Mycobacterium phlei* were also used. The fungi used were the yeasts, *Saccharomyces cerevisiae* and *Candida albicans*; the dermatophytic fungi *Trichophyton mentagrophytes* and *Microsporum gypseum*; and *Aspergillus fumigatus.* All cultures were standard laboratory strains from The University of British Columbia microbiological collection, Department of Microbiology.

An inoculum of each bacterial strain was suspended in 5 mL of Muller-Hinton broth (BBL) and incubated overnight at 37°C. The overnight cultures were diluted 1/10 with Muller-Hinton broth before use.

The fungal cultures were prepared by swabbing the parent plate with a cotton swab, and then transferring this to a vial containing 5 mL of Saboraud Dextrose broth (BBL).

Antibacterial/Antifungal Assays

The disc diffusion assay (Lennette, 1985) was used to screen for antibiotic and antifungal activity. A 100 µL aliquot of the diluted culture was spread on a sterile Muller-Hinton agar plate (BBL) (for bacteria) or a sterile Saboraud Dextrose agar (BBL) plate (for fungi). The impregnated discs were then placed on the plates and incubated for 30 min. to allow for diffusion. Chloramphenicol (for bacteria) or fungizone (for fungi) was used as a positive control, and MeOH was used as a negative control.

To test for light activated antibacterial/antifungal activity, one replicate was exposed to UV-A light (5 W/m^2, with a wavelength of 320-400 nm, from four Sylvania F20T12-BLB lamps) for 2 h while the other was kept in the dark. The plates were incubated for 18 h (48 h for *Mycobacterium*, and for varying times for the fungi) before the resulting zones of inhibition were observed and recorded. Tests were repeated twice times to ensure reliability of the results.

Results

All twenty-two of the extracts showed activity against at least two bacterial strains, and twenty-one showed activity against at least two fungi. Only four were active against Gram-negative bacteria. No extracts were active against

Pseudomonas aeruginosa or *Candida albicans* so these organisms were not included in the summary of the results, Tables 3.2 and 3.3.

The exposure to UV-A light had a considerable effect on the activities of some extracts, with four being active only when exposed to this light, and the antibacterial or antifungal effect of fifteen being enhanced upon exposure.

The extracts which exhibited the broadest spectra of activity (activity against at least five microorganisms) were: *Drymaria diandra* (Caryophyllaceae), aerial parts; *Macaranga pustulata* (Myrsinaceae), bark; *Corydalis longipes* (Fumariaceae), whole plant; *Hypericum cordifolium* (Hypericaceae), aerial parts; *H. elodeoides* (Hypericaceae), whole plant; *H. uralum* (Hypericaceae), whole plant; and *Maesa macrophylla*, bark.

The extracts that were light active were: *Drymaria diandra* against *Bacillus subtilis, Staphylococcus aureus, Streptococcus faecalis* and *Microsporum gypseum*; *Hypericum elodeoides* against *Serratia marcescens, H. uralum* against *Salmonella typhimurium,* and *Elsholtzia fruticosa* (Lamiaceae) against *Microsporum gypseum.*

Table 3.2

Antibacterial results of northern Nepalese plant extracts.

Family Species	Cat[b]	Pt[c]	B.s.[d]	S.a.	S.f.	E.c.	S.t.	S.m.	M.p.
Controls									
Methanol			-	-	-	-	-	-	-
Chloramphenicol			+	+	+	+	+	+	+
Asteraceae									
Centipeda minima	1,3	Ae	+*	+	-	-	-	-	+
Elephantopus scaber	3	Wh	+*	+*	-	-	-	-	+
Caryophyllaceae									
Drymaria diandra	1,3	Wh	+	+**	+**	-	-	-	+
Euphorbiaceae									
Macaranga pustulata	1,3	Bk	+**	+	+	-	-	-	+*
Fumariaceae									
Corydalis longipes	1	Wh	+**	+**	+**	-	-	-	+*
Gesneriaceae									
Corallodiscus lanuginosus	1,3	Wh	+**	-	-	-	-	-	+*
Didymocarpus primulifolius	3	Wh	+	-	-	-	-	-	+
Hypericaceae									
Hypericum cordifolium	1,3	Ae	+*	+	+	-	-	+**	+
Hypericum elodeoides	1,3	Wh	+	+	+	-	-	-	+
Hypericum uralum	1,3	Ae	+	+	+	-	+**	-	+
Lamiaceae									
Elsholtzia blanda	1,3	Ae	+*	-	-	-	-	-	-
Elsholtzia flava	1,3	Ae	+*	-	-	-	-	-	-
Elsholtzia fruticosa	1,3	Ae	+*	-	-	-	-	-	-
Micromera biflora	1,3	Wh	+	-	-	-	-	-	-
Pogostemon benghalensis	1,3	Ae	+	+	-	-	-	-	-
Lygodiaceae									
Lygodium japonicum	3	Wh	+	-	-	-	-	-	+
Myrcinaceae									
Maesa macrophylla	1	Bk	+	+	+	-	-	-	+*
Ranunculaceae									
Anemone obtusiloba	1,3	Rt	+	-	-	+	-	-	+
Rhamnaceae									
Zizyphus mauritiana	2	Rt	+	+	-	-	-	-	+
Rosaceae									
Princepia utilis	1,3	Lv	+	-	-	-	-	-	+
Sibbaldia micropetala	1	Wh	+*		-	-	-	-	+
Valerianaceae									
Valeriana jatamansii	1	Rt	+*	+	-	-	-	-	+

[a]Classification of results: -, no zone of inhibition; +, extract active; +*, activity enhanced by UV light; +**, active only when exposed to UV light.

[b]Cat, ethnomedicinal category: 1, potential antibiotic; 2, potential antifungal; 3, potential antiviral.

[c]Pt, Part extracted: Wh, Whole plant; Ae, Aerial parts; Lv, Leaves; Bk, Bark; Rt, Roots.

[d]Microorganisms: B.s., *Bacillus subtilis*; S.a., *Staphylococcus aureus* ; S.f., *Streptococcus faecalis*; E.c., *Escherichia coli*; S.m., *Serratia marcescens*; S.t., *Salmonella typhimurium*; P.a., *Pseudomonas aeruginosa*; M.p., *Mycobacterium phlei*.

Table 3.3

Antifungal results of northern Nepalese plant extracts.

Family Species	Cat[b]	Part[c]	S.c.[d]	M.p.	T.m.	A.f.
Controls						
Methanol			-	-	-	-
Fungizone			+	+	+	+
Asteraceae						
Centipeda minima	1,3	Ae	-	+	+	-
Elephantopus scaber	3	Wh	-	+	+	-
Caryophyllaceae						
Drymaria diandra	1,3	Wh	-	+	+	-
Euphorbiaceae						
Macaranga pustulata	1,3	Bk	-	+	+	-
Fumariaceae						
Corydalis longipes	1	Wh	+	+	+*	+
Gesneriaceae						
Corallodiscus lanuginosus	1,3	Wh	-	-	-	-
Didymocarpus primulifolius	3	Wh	-	+	+	-
Hypericaceae						
Hypericum cordifolium	1,3	Ae	-	+*	+*	-
Hypericum elodeoides	1,3	Wh	-	+*	+	-
Hypericum uralum	1,3	Ae	-	+*	+	-
Lamiaceae						
Elsholtzia blanda	1,3	Ae	+*	-	+	-
Elsholtzia flava	1,3	Ae	+*	-	+	-
Elsholtzia fruticosa	1,3	Ae	+	-	+*	-
Micromera biflora	1,3	Wh	+	-	+	-
Pogostemon benghalensis	1,3	Ae	+*	-	+	-
Lygodiaceae						
Lygodium japonicum	3	Wh	-	+	+	-
Myrcinaceae						
Maesa macrophylla	1	Bk	-	+	+	-
Ranunculaceae						
Anemone obtusiloba	1,3	Rt	-	+	+**	-
Rhamnaceae						
Zizyphus mauritiana	2	Rt	-	-	-	-
Rosaceae						
Princepia utilis	1,3	Lv	-	-	+	-
Sibbaldia micropetala	1	Wh	-	+	+	-
Valerianaceae						
Valeriana jatamansii	1	Rt	-	+	+	-

[a]Classification of results: -, no zone of inhibition; +, extract active; +*, activity enhanced by UV light; +**, active only when exposed to UV light.

[b]Cat, ethnomedicinal category: 1, potential antibiotic; 2, potential antifungal; 3, potential antiviral.

[c]Part extracted: Wh, Whole plant; Ae, Aerial parts; Lv, Leaves; Bk, Bark; Rt, Roots.

[d]Microorganisms: S.c., *Saccharomyces cervisiae*; M.p., *Microsporum gypseum* T.m., *Trichophyton mentagrophytes*; and A.f., *Aspergillus fumigatus*.

Discussion of Screening of Northern Nepalese Plant Extracts

A literature search of ethnobotanical information of the plants investigated showed that several are used by different tribes in different areas of Nepal to treat different ailments. The part of the plant and the way it is used varied, but the illness being treated could usually be interpreted as being caused by the same class of pathogen. For example, juice from *Drymaria diandra* is used to treat coughs, fever and eye disease (conjunctivitis) (Manandhar, 1989a,b; Shrestha and Joshi, 1993), which could all possibly be caused by bacterial pathogens. The methanolic extract of *D. diandra* was active against the Gram-positive bacteria tested.

The preliminary results obtained from the screening of the crude methanolic extracts indicated that further investigation was worth while. Several of the families and genera surveyed are known for their antibiotic properties. The light enhanced activity of the *Hypericum* species could very well be caused by the photodynamic compounds hypericin and related quinones (Ivie, 1982; Towers, 1984).

Members of the Fumariaceae (formerly included in Papaveraceae) are known to contain berberine-type alkaloids. Berberine is phototoxic (Philogene et al., 1984), and either this compound or related alkaloids may be responsible for the photoactivity of the *Corydalis longipes* extract. The light enhanced activity of *Elephantopus scaber* L.(Asteraceae), and *Centipeda minima* (L.) A. Br. & Aschers (Asteraceae) may be attributed to the presence of light activated polyynes which are common in species of the Asteraceae (Bohlman et al., 1973; Towers, 1980). A number of chalcones, quinochalcones and flavanones have been isolated from *Didymocarpus pedicellata*, some of which show antifungal activity (Guha and Bhattacharyya, 1992). These compounds may

also be present in *Didymocarpus primulifolius*, which was found to be active against *Candida albicans*; and *Trichophyton mentagrophytes*.

Antimicrobial Screens of Southern Nepalese Medicinal Plants

Methodology

Extract and Disc Preparation

The extracts were prepared according to the procedure previously described in the screening of northern Nepalese medicinal plants. The only exception was that the extracts were diluted in MeOH to a concentration of 50 mg/mL. Paper discs were impregnated with 20 μL of this extract (corresponding to 1 mg of crude plant extract) and allowed to dry at room temperature.

Microorganisms

Eleven strains of bacteria and four strains of fungi were used in the screening process. The bacterial strains that have not been previously described in Chapter III are methicillin resistant and sensitive *Staphylococcus aureus*, Gram-negative *Klebsiella pneumoniae, Enterobacter aerogenes,* and super sensitive (to all antibiotics tested) *Pseudomonas aeruginosa*. All cultures were standard laboratory strains from The University of British Columbia Microbiological collection, except the strains of *Staphylococcus,* and *Pseudomonas aeruginosa*; which were gifts of Dr. R. Hancock, Department of Microbiology, The University of British Columbia.

Cultures were prepared as previously described in the screening of northern Nepalese medicinal plants.

Antibacterial/Antifungal Assays

The same method was used to test for antibiotic/antifungal activities of the southern plants as was used for the northern plants, with the following

exceptions. Gentamicin (for bacteria) or nystatin (for fungi) was used as a positive control, and MeOH was used as a negative control. Gentamicin was administered at 10 μg per disk, while nystatin was applied at 25 μg per disk. Methanol was introduced in the same amount as the extracts, 20 μL per disk.

Results

Fifteen of the twenty extracts tested showed activity against bacteria and fourteen showed activity against fungi, either full activity or spore inhibition. Only four were active against Gram-negative bacteria. None of the extracts was active against *Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes, Salmonella typhimurium* or *Aspergillus fumigatus*, so these organisms were not included in the summary of results (Tables 3.4 and 3.5).

The exposure to UV-A light had a considerable effect on the activities of some extracts, with four extracts being active only when exposed to light. The antibacterial and antifungal effects of four extracts were enhanced upon exposure.

The extracts which exhibited the broadest spectra of activity (activity against at least five microorganisms) were: *Eupatorium odoratum* (Asteraceae), *Terminalia alata* (Combretaceae), *Mallotus philippensis* (Euphorbiaceae), and *Rumex hastatus* (Polygonaceae).

The extracts that were light active, or light enhanced, were: *Scindapsus officinalis* (Araceae), *Eupatorium odoratum, Terminalia alata, Mallotus philippensis, Streblus asper,* (Moraceae), and *Lippia nodiflora*, (Verbenaceae).

Table 3.4

Antibacterial activities of southern Nepalese plant extracts.

Family Species	Cat[b]	Pt[c]	Bs[d]	Sa MS	Sa MR	Sf	Pa 188	Pa 187	Mp
Controls									
Methanol			-	-	-	-	-	-	-
Gentamycin			+	+	-	-	+	-	+
Acanthaceae									
Peristrophe bicalculata	1,3	Ae	-	-	-	-	-	-	-
Rungia parviflora	1,3	Ae	-	-	-	-	-	-	+
Amaranthaceae									
Alternanthera sessilis	1,3	Ae	-	-	-	-	-	-	-
Apocynaceae									
Carissa carandas	1,3	Rt	-	-	-	-	-	-	-
Araceae									
Scindapsus officinalis	1,3	Ft	-	+**	-	-	-	-	-
Asteraceae									
Eupatorium odoratum	1	Ae	+	+	+	-	-	-	+*
Inula cappa	1,3	Rt	-	+	-	-	-	-	-
Tridax procumbens	1	Ae	-	-	-	-	-	-	-
Combretaceae									
Terminalia alata	1,3	Bk	+	+*	+	+	+	+	+
Euphorbiaceae									
Mallotus philippensis	1,3	Bk	+	+	+*	-	+**	-	+
Jatropha gossipifolia	1,3	Bk	-	-	-	-	-	-	-
Fabaceae									
Bauhinia vahlii	1	Rt	+	+	+	+	+	-	+
Millettia extensa	1	Rt	-	-	-	-	-	-	+
Millettia extensa	1	Lv	+**	-	-	-	-	-	+
Malvaceae									
Sida cordata	1	Rt	-	-	-	-	-	-	+
Urena lobata	1,3	Sd	-	-	-	-	-	-	-
Moraceae									
Streblus asper	1,3	Bk	-	+**	+**	-	-	-	-
Polygonaceae									
Rumex hastatus	1	Rt	+	+	+	+	+	+**	+
Rutaceae									
Aegle marmelos	1,3	Rt	-	-	-	-	-	-	+
Verbenaceae									
Clerodendron indicum	1,3	Ae	+	-	-	-	-	-	-
Lippia nodiflora	1,3	Ae	-	+*	+*	-	-	-	+

[a]Classification of results: -, no zone of inhibition; +, extract active; +*, activity enhanced by UV light; +**, active only when exposed to UV light.

[b]Cat, ethnomedicinal category: 1, potential antibiotic; 2, potential antifungal; 3, potential antiviral.

[c]Pt, Part extracted: Wh, Whole plant; Ae, Aerial parts; Lv, Leaves; Bk, Bark; Rt, Roots; Sd, Seeds.

[d]Microorganisms: Bs, *Bacillus subtilis*; SaMS, *Staphylococcus aureus* - Methicilin sensitive; SaMR *Staphylococcus aureus* - Methicilin resistant; Sf, *Streptococcus faecalis*; Pa188, *Pseudomonas aeruginosa* - Sensitive; Pa187, *Pseudomonas aeruginosa* -Wild type; Mp, *Mycobacterium phlei*.

Table 3.5

Antifungal activities of southern Nepalese plant extracts.

Family Species	Cat[b]	Part[c]	S.c.[d]	C.a.	T.m.
Controls					
Methanol			-	-	-
Nystatin			+	+	+
Acanthaceae					
Peristrophe bicalculata	1,3	Ae	-	-	-
Rungia parviflora	1,3	Ae	-	-	s
Amaranthaceae					
Alternanthera sessilis	1,3	Ae	-	-	s
Apocynaceae					
Carissa carandas	1,3	Rt	-	-	s
Araceae					
Scindapsus officinalis	1,3	Ft	-	-	+
Asteraceae					
Eupatorium odoratum	1	Ae	-	-	s
Inula cappa	1,3	Rt	-	-	s
Tridax procumbens	1	Ae	-	-	s
Combretaceae					
Terminalia alata	1,3	Bk	+	+	s
Euphorbiaceae					
Mallotus philippensis	1,3	Bk	-	-	-
Jatropha gossipifolia	1,3	Bk	-	-	-
Fabaceae					
Bauhinia vahlii	1	Rt	-	-	-
Millettia extensa	1	Rt	-	-	-
Millettia extensa	1	Lv	-	-	s
Malvaceae					
Sida cordata	1	Rt	-	-	+*
Urena lobata	1,3	Sd	-	-	s
Moraceae					
Streblus asper	1,3	Bk	-	-	-
Polygonaceae					
Rumex hastatus	1	Rt	-	-	s
Rutaceae					
Aegle marmelos	1,3	Rt	-	-	+
Verbenaceae					
Clerodendron indicum	1,3	Ae	-	-	-
Lippia nodiflora	1,3	Ae	-	-	-

[a]Classification of results: -, no zone of inhibition; +, extract active; +*, activity enhanced by UV light; +**, active only when exposed to UV light, s, extract inhibits spore production.

[b]Cat, ethnomedicinal category: 1, potential antibiotic; 2, potential antifungal; 3, potential antiviral.

[c]Part extracted: Wh, Whole plant; Ae, Aerial parts; Lv, Leaves; Bk, Bark; Rt, Roots; Sd, Seeds.

[d]Microorganisms: S.c., *Saccharomyces cervisiae*; C.a., *Candida albicans*; T.m., *Trichophyton mentagrophytes.*

Discussion of Screening of Southern Nepalese Plant Extracts

The methodology of this second screening was modified somewhat from the first screening in order to allow for results to be compared with greater ease, and to take into account the greater number of microorganisms that were available for screening purposes. The extracts were all standardized using a set concentration of crude extract. This differed slightly from the first study which used a set amount of dried plant material, a standard qualitative technique in the Towers laboratory (McCutcheon et al., 1993). This modification allowed for comparison of antibacterial and antifungal activity with results from other assays. This is especially necessary for comparisons with antiviral assays, where a high concentration of crude extract is likely to be cytotoxic, so that using an exact concentration is necessary. It also allowed comparison of results with other antibacterial and antifungal screens taking place in the laboratory at the same time (see Jovel, 1996; Mutta, 1996). The screening organisms were augmented with two sensitive species, and one resistant strain. This made the screening even more relevant, as the methicillin resistant *Staphylococcus aureus* is a dangerous hospital pathogen.

The preliminary results obtained from the screening of the crude methanolic extracts indicated that further investigation was worthwhile. *Terminalia* species are known to contain tannins (e.g. Tanaka et al., 1991), which could be responsible for the antibiotic activity shown in the *Terminalia alata* extract. Terpenoid compounds are also known to be present in the methanolic fractions of some *Terminalia* species (e.g. Kundu and Mahato, 1993) but it is not known whether these compounds possess any antibiotic activity. *Eupatorium odoratum* was found by Valsaraj and coworkers (1996) to be active against both Gram-positive and Gram-negative bacteria, while in this study it was found to be active only against Gram-positive bacteria. This could

be because Valsaraj et al. used a concentration of 6.25 mg/mL. The light enhanced activity of *Eupatorium odoratum* in my screening may be attributed to the presence of photosensitizing polyynes which are common in species of the Asteraceae (Bohlman et al., 1973; Towers, 1980), while the light activity of *Rumex hastatus* could be ascribed to the presence of hydroxyanthraquinones, which are known to be present in members of the Polygonaceae, including species of *Rumex* (Thomson, 1971). *Streblus asper* was also light active, which may be because of furanocoumarins, which are known to be present in other species in the Moraceae (Murray et al., 1982).

The medicinal information that *T. alata* is used to treat diarrhoea and dysentery was provided by Kamalsing Raute. The Raute have a strong bond with the forest, and it would be interesting to investigate their herbal knowledge further, as this information gave rise to the discovery that *T. alata* possesses such a broad range of antimicrobial activity. Kamalsing Raute also gave information about the use of *Rungia parviflora* which was only slightly active against two microorganisms, *Trichophyton mentagrophytes* and *Mycobacterium phlei.* While *T. alata* is used to treat diarrhoea, a likely symptom of bacterial infection, *R. parviflora* is used to treat fever. Fever could indicate a bacterial or viral infection, and a herbal drug used to treat fever could either combat the infection that is resulting in the fever, or reduce the body temperature of the patient.

General Discussion

There are several plants tested in this screening which had been inactive in other screenings performed in the sixties and seventies. One such species

was *C. minima*, which had been reported by Bhakuni and coworkers (1969) to lack antibiotic activity, but to display activity against the protozoan, *Entamoeba histolytica*. It was also assayed by Lin and coworkers (1973) for antibacterial activity and found to be inactive. The cause of this discrepancy may be that older bioassays were not sensitive enough (not enough bacterial strains used, or perhaps not a high enough concentration of plant extract used) and therefore plants that were considered 'inactive' 20 years ago are now known to be 'active'.

There are several factors which must be taken into account when discussing the activities of medicinal plants. One obvious problem is that the plant extract tested in the laboratory is not prepared in the same way as the medicine taken in the field (Prance, 1994). Unfortunately, in my screening this could not be avoided because there were no testing facilities in Nepal, and the plant material had to be dried to transport it into Canada. In the north of Nepal, healers use dried plants frequently, mainly because of the short growing season. In the south, however, the healers prefer fresh plants, and activity may have been lost in the drying and transportation processes. This may account for the fact that 100% of the northern plants had some antimicrobial activity, while only 75% of the southern plants tested positive.

Ecology, soil, climate, diurnal and other temporal factors affect secondary metabolite production in the plants (Prance, 1994), and this could affect the concentration or presence of certain biologically active components. This may have been a factor in the absence of *in vitro* activity of some of the medicinal plants collected, although advice given by the healers as to where and when to collect the plants was heeded.

Plants taken to treat symptoms of infectious diseases may work in many ways besides acting directly on the invading pathogen. They may lower fever,

boost the immune system, or act as a painkiller. None of these effects would be seen in the *in vitro* disk diffusion assays done in this screening. Also, plants used to treat ailments such as sore throats and coughs may be taken for their soothing properties (much like cough drops). While they do nothing to combat infection directly, they do make the patient feel better.

The plants were extracted with methanol because it is an inexpensive, common solvent that can be removed easily. Methanol extracts a range of phytochemicals, but may not dissolve the very lipophilic components or the very polar ones such as peptides. These components could be extracted by using another solvent, but as Farnsworth (1994) stated at the 1994 CIBA Foundation Symposium on Ethnobotany and the Search for New Drugs, for every extra solvent used to extract, the cost of the testing doubles. For this reason it was decided to use methanol as the solvent in this study.

Farnsworth (1994) believes that approximately 90% of the time traditional healers use boiling or hot water in to make herbal extracts, yet in many cases the active compounds are not water soluble. He suggests that most likely there are saponins or other compounds in the plant that affect surface tension and solubilize the biologically active compounds. Crude aqueous extracts are really colloidal systems hence non-polar compounds will appear to be polar in them. Once these solubilizing components have been removed the active ingredient is in a different physical environment and behaves accordingly. This will be discussed further in the following chapters on isolation and identification of active compounds.

The disc diffusion assay used in this screening was chosen for speed and reproducibility. In order to assess the antimicrobial activity of purified compounds, a more quantitative approach is needed. In the following chapters, purified compounds were assayed by the minimum inhibitory concentration

(MIC) method. This method indicated if the compound was bactericidal or merely bacteriostatic.

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Chapter IV

Antiviral screening

Introduction

Viruses are obligatory intracellular parasites, containing a single type of nucleic acid (either DNA or RNA). They are surrounded by a protein coat, measure only 20 - 300 nm in diameter, and multiply only inside living cells, using the synthetic machinery of the cell (Melnick, 1978). The latter makes the quest for antiviral drugs a difficult one. Most compounds that would inhibit viral multiplication would also interfere with host cell function and therefore would be toxic to the organism being treated.

Not surprisingly, viruses have resisted prophylaxis or therapy longer than any other form of life (Vanden Berghe et al., 1986). Infectious viral diseases are a world-wide problem. In Europe and North America, it is estimated that at least 60% of infectious illnesses are viral in nature, yet as of 1988 there were only six antiviral drugs approved for use in the USA (Tortora et al., 1989). This number should steadily increase over the next few years with the push to develop drugs to treat human immunodeficeincy virus (HIV).

There are several steps to the multiplication of a virus, with each one offering an opportunity to disrupt the cycle with an antiviral drug. The virus must attach itself to the host cell, penetrate the cell membrane, uncoat, duplicate its genetic material, assemble and be released from the infected cell. A more detailed explanation of the viral 'life cycle' can be seen in Vanden Berghe et al. (1986, page 103). Antiviral drugs may interrupt this 'virus life cycle', or may act directly on the virus (virucidal activity).

A good antiviral therapeutic compound should act specifically on the virus and not damage the host cell. This poses a problem for antiviral researchers, as viruses may remain latent in a host cell for some time. Antiviral drugs (seen in Figure 4.1) include amantadine, which acts on the influenza A virus by preventing penetration. 5-Iodo-2'-deoxyuridine (Idoxuridine, or IdU), which is active against herpes simplex virus (HSV), cytomegalo virus (CMV), varicella-zoster virus (VZV) and adenovirus, competitively inhibits enzymes involved in DNA replication, and prevents synthesis of mature virus. 9-((2-Hydroxyethoxy)methyl)guanine (acyclovir) is active against a range of herpesviruses, including HSV, CMV, VZV, and Epstein Barr virus (EBV). Acyclovir is phosphorylated and incorporated into the 3' terminus of elongating DNA, which prevents complete DNA replication. Even if a drug is too cytotoxic to be a useful therapeutic agent, it may have some use in the study of viral replication.

There have been many studies published on plant extracts and purified phytochemicals that show antiviral activity (for examples of work conducted in the Towers and Hudson laboratories see Hudson et al., 1991, 1993a,b; MacRae et al., 1988; Yip et al., 1991, 1995). Several compounds that represent a range of plant chemicals and antiviral activities can be seen in Figure 4.2, with activities and sources documented in Table 4.1 (Vanden Berghe et al., 1986; Hudson, 1990).

Vanden Berghe's group (Vanden Berghe et al., 1986) found that plants collected on the basis of traditional medicinal usage yielded five times the active number of species compared to random screenings . In some cases the same phytochemical was responsible for the antiviral activity of several plant species. Vanden Berghe et al. (1986) have defined a procedure for the screening of plants for antiviral activity, which is very similar to the procedure

followed in my screening. They suggest using a variety of viruses that represent the major viral categories (DNA and RNA viruses, enveloped and non-enveloped), but state that the viral cytopathic effects (CPE) should be visible within a week to make the screen efficient. This was taken into consideration when designing my screen.

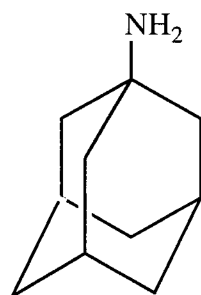
The viruses used in the antiviral assays were all animal viruses. Two are enveloped viruses; Sindbis (SINV) and herpes simplex virus 1 (HSV). Sindbis is a single-stranded (+) RNA (11,000 nucleotides in length) virus of the togaviridae with an icosahedral capsid of diameter ~40 nm. Disease causing members of the togaviridae include the rubella virus (German measles), yellow fever virus, dengue fever virus and many viruses causing encephalitis. Herpes simplex virus is a double stranded DNA (approximately 170,000 nucleotides in length) virus of the herpesviridae with an icosahedral capsid having a diameter of ~180 nm. Besides herpes, disease caused by viruses of the herpesviridae include chicken pox, infectious mononucleosis and shingles.

The non-enveloped virus used was human polio virus 1 (POLIO), a single-stranded (+) RNA (approximately 8,000 nucleotides in length) virus in the picornaviridae, which has an icosahedral capsid with a diameter of ~25 nm. Picorna viruses are the smallest RNA viruses, taking their name from 'pico' meaning small, and RNA. Viruses of the picornaviridae include hepatitis A virus, viruses which cause enteritis, and rhinoviruses, which are the most common cause of colds .

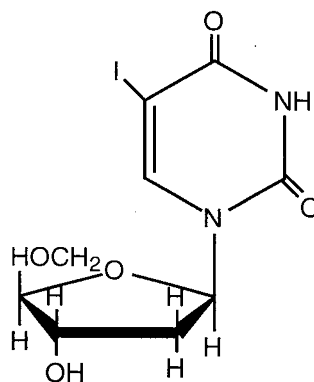
Plant extracts and phytochemicals are becoming popular as potential sources of antivirals, and several reviews have been written (Vanden Berghe et al., 1986; Hudson, 1990, 1995, Hudson and Towers, 1991). By using the ethnobotanical knowledge of the indigenous Nepalese people as a means to

select potentially antiviral plants, this screening has the potential to be more successful than a random screening of Nepalese plants.

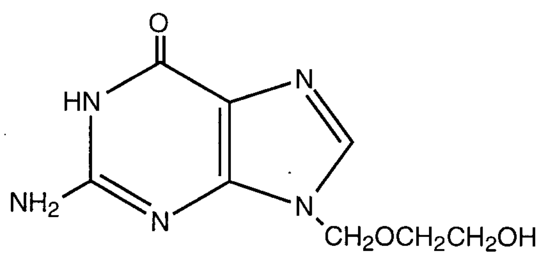
The antiviral assays included tests in UV-A and visible light. This is necessary because many natural antivirals are photosensitizers (Hudson and Towers, 1991), e.g. hypericin, which is significantly more active against the AIDS virus (HIV-1) in light than in dark (Hudson et al., 1993b). Photosensitizers are discussed more thoroughly in Chapter III.



Amantadine

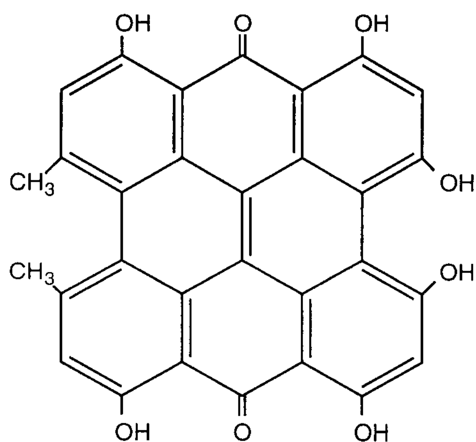


Idoxuridine

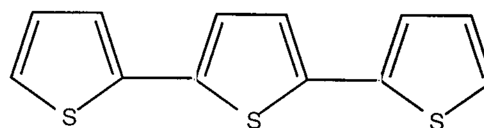


Acyclovir

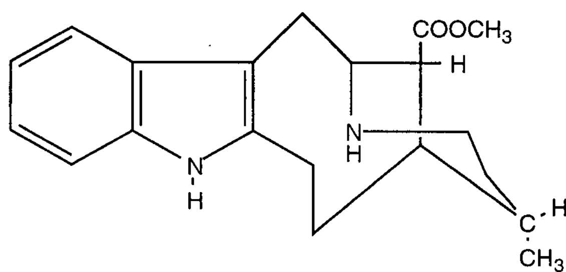
Figure 4.1. Some synthetic antiviral drugs



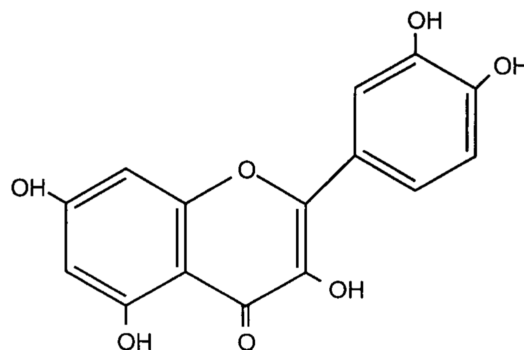
Hypericin



α-Terthienyl



Perivine



Quercetin

Figure 4.2. Some antiviral phytochemicals

Table 4.1

Some antiviral phytochemicals, with source plant and activities.

Compound	Source Plant (Family)	Target Virus	Reference
Hypericin	*Hypericum perforatum* L.	HIV	Hudson et al., 1993b; Lenard et al., 1993; Lavie et al., 1989
		HSV	Tang et al., 1990
		SINV, MCMV (murine cytomegalo virus)	Hudson et al., 1991
Perivine	*Catharanthus roseus* (L.) G. Don. (Apocynaceae)	vaccinia POLIO	Farnsworth et al., 1968
α-Terthienyl	*Chaenactis douglasii* (Asteraceae)	HIV	Hudson et al., 1993a
		SINV, MCMV	Hudson et al., 1993d
Quercetin	Widely distributed	HSV Prophylactic against rabies in mice when given in diet.	Vanden Berghe et al., 1986

Methodology

Extract Preparation

The methanolic extracts that had been previously prepared for the antimicrobial screening were dissolved in Hanks medium (1X) to give a concentration of 400 μg/mL of crude plant extract. A final concentration of MeOH no greater than 1% in Hanks was always maintained.

Cell Culture and Cytotoxicity Assays

The cell line used was Vero, an African green monkey kidney cell line. The cells were grown in monolayer in a 5% carbon dioxide and 95% air atmosphere at 37 °C, in Dulbecco's Modified Eagle medium (MEM) with 10% fetal bovine serum (FBS) (GIBCO-Life Sciences, Ontario) and 25 μg/mL gentamicin sulphate (Sigma).

To test for cytotoxicity, Vero cell monolayers were grown in 96 well microtiter plates (Falcon 3072), and exposed to serial dilutions of the extracts, starting at 200 μg/mL crude plant. The treated cells were then incubated at 37 °C for one hour, exposed to either UV-A light (5 W/m^2, with an emission wavelength maximum of 350 nm, from four Sylvania F20T12-BLB lamps), visible light (5 W/m^2, with an emission wavelength maximum of 580 nm, from four Westinghouse 20 W fluorescent tubes (F20T12/cw)), or kept in the dark, for 30 min., and then re-incubated for 24 hours. The cells were examined microscopically for the presence of cytotoxic effects, which were noted in Tables 4.2 and 4.3.

Antiviral Assays

To screen for antiviral activity, Vero cell monolayers were grown in 96 well microtiter plates. The extracts were added in two-fold serial dilutions

starting at 200 μg/mL (as in the cytotoxicity assays). One hundred plaque forming units (PFU) of virus were added to each well after the one hour incubation period, before exposure to any light treatments. The cultures were then incubated for a suitable amount of time to allow development of cytopathic effects (CPE); 24 hours for POLIO, 48 hours for SINV, and 96 hours for HSV. When infected with polio virus, Vero cells shrink and adopt a distinct crescent moon shape. SINV tends to make Vero cells shrink, while HSV-1 infection is characterized by rounding, swelling and bunching of the Vero cells, so they resemble bunches of grapes. Controls consisted of cells only and cells incubated with untreated virus. Antiviral activity was indicated by a total inhibition of viral CPE at a non-cytotoxic concentration of extract. Partial inhibition of the virus (a marked reduction in infectivity of the virus exposed to an extract when compared to the untreated virus control) was also recorded. Acyclovir was used as a positive control against herpes simplex virus. It was a gift from Dr. J. Sassadeus, UBC Hospital. It was already dissolved in media at a concentration of 225 μg/mL. Acyclovir gave 100% inhibition at 1.8 μg/mL, with partial inhibition at 0.5 μg/mL.

Table 4.2

Cytotoxicity levels of the northern Nepalese plant extracts.

Family Species	Medicinal category[a]	Part used in treatment[b]	Cytotoxicity[c] (μg/mL.)
Asteraceae			
Centipeda minima	1,3	Ae	25
Elephantopus scaber	3	Wh	200
Caryophyllaceae			
Drymaria diandra	1,3	Wh	-
Euphorbiaceae			
Macaranga pustulata	1,3	Bk	-
Fumariaceae			
Corydalis longipes	1	Wh	-
Gesneriaceae			
Corallodiscus lanuginosus	1,3	Wh	-
Didymocarpus primulifolius	3	Wh	-
Hypericaceae			
Hypericum cordifolium	1,3	Ae	200
H. elodeoides	1,3	Wh	-
H. uralum	1,3	Wh	-
Lamiaceae			
Elsholtzia blanda	1,3	Ae	-
E. flava	1,3	Ae	-
E. fruticosa	1,3	Ae	-
Micromeria biflora	1,3	Wh	200
Pogostemon benghalensis	1,3	Ae	-
Lygodiaceae			
Lygodium japonicum	3	Wh	-
Myrsinaceaea			
Maesa macrophylla	1	Bk	200
Ranunculaceae			
Anemone obtusiloba	1,3	Rt	200
Rhamnaceae			
Zizyphus mauritiana	2	Rt	-
Rosaceae			
Princepia utilis	1,3	Lv	-
Sibbaldia micropetala	1	Wh	-
Valerianaceae			
Valeriana jatamansii	1	Rt	-

[a]Cat, ethnomedicinal category: 1, potential antibiotic; 2, potential antifungal; 3, potential antiviral.

[b]Part extracted: Wh, Whole plant; Ae, Aerial parts; Lv, Leaves; Bk, Bark; Rt, Roots.

[c]Cytotoxicity: lowest concentration of extract to produce a cytotoxic effect. Highest concentration tested was 200 μg/mL, - indicates no cytotoxicity

Table 4.3

Cytotoxicity levels of the southern Nepalese plant extracts.

Family Species	Medicinal category[a]	Part used in treatment[b]	Cytotoxicity[c] (μg/mL.)
Acanthaceae			
Peristrophe bicalyculata	1,3	Wh	-
Rungia parviflora	1,3	Wh	-
Amaranthaceae			
Alternanthera sessilis	1,3	Wh, Ae	-
Apocynaceae			
Carissa carandas	1	Rt	25
Araceae			
Scindapsus officinalis	1	Fr	-
Asteraceae			
Eupatorium odoratum	1	Ae	200
Inula cappa	1,3	Rt	-
Tridax procumbens	1	Wh	-
Combretaceae			
Terminalia alata	1	Bk	200*
Euphorbiaceae			
Mallotus philippensis	1	Bk	-
Jatropha gossipifolia	1,3	Bk	-**
Fabaceae			
Bauhinia vahlii	1	Rt	100
Milettia extensa	1,4	Rt	50
		Ae	200
Malvaceae			
Sida cordata	1,2,3	Rt	-
Urena lobata	1	Sd	-
Moraceae			
Streblus asper	1	Bk	200
Polygonaceae			
Rumex hastatus	1,3	Rt	100
Rutaceae			
Aegle marmelos	1,3	Rt	-
Verbenaceae			
Clerodendrum indicum	1,3	Wh	-
Lippia nodiflora	3	Wh	-

[a]Cat, ethnomedicinal category: 1, potential antibiotic; 2, potential antifungal; 3, potential antiviral; 4, potential insecticidal.

[b]Part extracted: Wh, Whole plant; Ae, Aerial parts; Lv, Leaves; Bk, Bark; Rt, Roots; Fr, Fruits; Sd, Seeds.

[c]Cytotoxicity: lowest concentration of extract to produce a cytotoxic effect. Highest concentration was 200 μg/mL.

*many cytoplasmic vacuoles present at 25-100 μg/mL but not apparently cytotoxic.

**many cytoplasmic vacuoles present at 25-200 μg/mL but not apparently cytotoxic.

Results (northern Nepalese medicinal plants)

Seventeen out of the twenty-two plant species collected are used traditionally to treat diseases that could be caused by viral pathogens, for example coughs, colds, influenza, fever, herpes lesions and chicken pox. Of these seventeen species, thirteen of the extracts were at least partially antiviral, with five totally inactivating at least one virus. Sixteen of the twenty-two medicinal extracts showed at least partial antiviral activity, while seven were 100% active. The results of the most active extracts are listed in Table 4.4, with partial activity listed in Table 4.5. The active concentrations ranged from 13-200 μg/mL, with 200 μg/mL the highest concentration of extract tested. It is possible that other extracts may be active at a higher concentration, as many extracts were not cytotoxic at 200 μg/mL. Plant extracts tested that were not active were: *Corydalis longipes* (Fumariaceae), *Hypericum elodeoides* (Hypericaeae), *Elsholtzia flava*, (Lamiaceae), *E. fruticosa* (Lamiaceae), *Micromera biflora* (Lamiaceae), and *Zizyphus mauritiana* (Rhamnaceae).

Table 4.4

Northern Nepalese plants: most active extracts[a].

Family Species	SINV[b]	POLIO	HSV
Caryophyllaceae			
Drymaria diandra	200 UV only	-	-
Euphorbiaceae			
Macaranga pustulata	200	100 (partial) UV only	200
Hypericaeae			
Hypericum cordifolium	100	-	50
H. uralum	200 (partial), 200 UV only	100 (partial), UV only	200, 200 (partial) VIS only
Lygodiaceae			
Lygodium japonicum	200 (partial) 50 VIS only, 100 UV only	-	-
Myrcinaceae			
Maesa macrophylla	100 (partial)	100, 100 (partial) UV only	100
Rosaceae			
Sibbaldia micropetala	-	200 (partial)	200, 100 VIS only

[a]Classification of results: -: no antiviral activity; 100: extract inactivates virus at 100 µg/mL in dark; (partial) extract partially inactivates virus at concentration given; VIS only: extract only active at concentration given in the presence of visible light; UV only: extract only active at concentration given in the presence of UV-A radiation.

[b]Viruses: SINV, Sindbis virus; POLIO, Human poliovirus 1; HSV, herpes simplex virus-1.

Table 4.5

Northern Nepalese plants: partially active extracts[a].

Results are listed as lowest concentration of extract (µg/mL) able to partially inhibit the virus.

Family Species	SINV[b]	POLIO	HSV
Asteraceae			
Centipeda minima	13	13	13
Elephantopus scaber	100	200 (UV only)	-
Gesneriaceae			
Corallodiscus lanuginosus	-	-	100 (VIS only)
Didymocarpus primulifolius	200, 100 (UV only)	-	
Lamiaceae			
Elsholtzia blanda	100 (UV only)	-	-
Pogostemon benghalensis	200	-	-
Ranunculaceae			
Anemone obtusiloba	200, 100 (VIS only)	100	100
Rosaceae			
Princepia utilis	-	200	200 (VIS and UV only)
Valerianaceae			
Valeriana jatamansii	-	100	-

[a]Classification of results: -: no antiviral activity; 100: extract partially inactivates virus at 100 µg/mL in dark; VIS only: extract only active at concentration given in the presence of visible light; UV only: extract only active at concentration given in the presence of UV-A radiation.

[b]Viruses: SINV, Sindbis virus; POLIO, Human poliovirus 1; HSV, herpes simplex virus-1.

Discussion (Northern Nepalese Medicinal Plants)

Two of the three *Hypericum* species tested were active, one against all three test viruses, and one only against the membrane bound viruses, Sindbis virus and herpes simplex virus. This activity was not light enhanced which would suggest the presence of antiviral compounds other than hydroxyanthraquinones (such as hypericin) that have not yet been identified. Only one of the *Elsholtzia* species tested had antiviral activity. *E. blanda* was partially active against Sindbis virus only when exposed to UV-A radiation. Several *Elsholtzia* species (Lamiaceae) have been documented to have antiviral activity against Sindbis virus when exposed to UV-A light (Yip, 1993). These include *Elsholtzia ciliata* (Thunb.) Hylander, which was active at 30 µg/mL, *E. splendens* Nakai ex F. Maek, and *E. blanda*. The photoactivity of *E. ciliata* was found to be caused in part by the polycyclic aromatic, fluoranthene. Fluoranthene has not been documented in plants before, and is almost certainly an environmental pollutant (Yip, 1993). Another *Elsholtzia* species, *E. densa* Briq, was determined to be antiviral, but not photoactive (Yip, 1993).

The virus most resistant to the extracts was poliovirus, with only one extract, that of *Maesa macrophylla*, inactivating it completely. *M. macrophylla* was active against the other two viruses as well. Poliovirus was the only virus tested that was not membrane bound. Poliovirus is very stable, remaining infectious for relatively long periods in water or food (Hudson, 1990). This stability and lack of membrane probably accounts for the resistance to the medicinal plant extracts.

Lygodium japonicum, which is used to treat genital and cold sore herpes in Nepal, was not active against herpes simplex virus in this screening. It was, however, active against the membrane bound SINV; its activity increasing with

exposure to UV-A or visible light. This clearly demonstrates the importance of testing for light mediated activity when screening plant extracts. It also demonstrates the difficulty in determining the exact illness a traditional remedy is used to treat. The symptoms of herpes are much more difficult to describe than a cough or sore throat, and perhaps this herb is really used to treat a disease that was interpreted as herpes, but is actually something else.

Elephantopus scaber was active against poliovirus only in the presence of UV-A radiation. *E. scaber* is a member of the Asteraceae, a family known to contain photoactive polyynes (Bohlman et al., 1973; Towers, 1980). This activity is unusual, as many photoactive compounds only are active on membrane bound viruses, but this extract was only active on the non-enveloped virus, and only in the presence of UV-A light.

Results (Southern Nepalese Medicinal Plants)

Nine out of the 21 plant extracts (nine out of the 20 plant species) tested were used traditionally to treat diseases that could be caused by viral pathogens, for example coughs, colds, and fever. Of these nine plants, six of the extracts were at least partially antiviral, with one totally inactivating at least one virus. Fourteen of the medicinal extracts showed at least partial antiviral activity, while eight were 100% active. The results of the most active extracts are listed in Table 4.6, with partial activity listed in Table 4.7. The active concentrations ranged from 3-200 μg/mL, with 200 μg/mL the highest concentration of extract tested. It is possible that other extracts may be active at a higher concentration, as many extracts were not cytotoxic at 200 μg/mL. Plant extracts tested that were not active were: *Clerodendrum indicum* (Verbenaceae), *Peristrophe bicalyculata* (Acanthaceae), *Rungia parviflora*

(Acanthaceae), *Scindapsus officinalis* (Araceae), *Sida cordata* (Malvaceae) and *Urena lobata* (Malvaceae).

Table 4.6

Most active extracts[a] of southern Nepalese plants.

Minimum inhibitory concentrations given in µg/mL.

Family Species	SINV[b]	POLIO	HSV
Asteraceae			
Tridax procumbens	Partial: 200	Partial: 12	200 Partial: 25 (dark and UV), 50 (Vis)
Apocynaceae			
Carissa carandas	3	6 Partial: 3	12 Partial: 3
Euphorbiaceae			
Mallotus philippensis	Partial: 200	Partial: 50	100 Partial: 25 (dark and Vis), 50 (UV)
Milettia extensa (Root)	25 Partial: 12	-	-
Milettia extensa (Aerial)	Partial: 100	Partial: 100	100
Fabaceae			
Bauhinia vahlii	-	-	25
Moraceae			
Streblus asper	100 Partial: 50	Partial: 50	50
Polygonaceae			
Rumex hastatus	-	-	50 Partial: 25
Combretaceae			
Terminalia alata	100	Partial: 50	25

[a]Classification of results: -: no antiviral activity; 100: extract inactivates virus at 100 µg/mL in dark; partial: extract partially inactivates virus at concentration given; Vis: extract only active at concentration given in the presence of visible light; UV: extract only active at concentration given in the presence of UV-A radiation.

[b]Viruses: SINV, Sindbis virus; POLIO, Human poliovirus 1; HSV, herpes simplex virus-1.

Table 4.7

Partially active extracts[a] of southern Nepalese plants.

Results are listed as lowest concentration of extract (µg/mL) able to partially inhibit the virus.

Family Species	SINV[b]	POLIO	HSV
Asteraceae			
Eupatorium odoratum	-	200	-
Inula cappa		25	
Amaranthaceae			
Alternanthera sessilis	-	-	200 (UV)
Euphorbiaceae			
Jatropha gossipifolia	200	-	200 (Vis)
Rutaceae			
Aegle marmelos	-	-	200
Verbenaceae			
Lippia nodiflora	-	-	200

[a]Classification of results: -: no antiviral activity; 200: extract partially inactivates virus at 200 µg/mL in dark; Vis: extract only active at concentration given in the presence of visible light; UV: extract only active at concentration given in the presence of UV-A radiation.

[b]Viruses: SINV, Sindbis virus; POLIO, Human poliovirus 1; HSV, herpes simplex virus-1.

Discussion (Southern Nepalese Medicinal Plants)

The methanol extract of *Carissa carandas* was very active against all three viruses. This activity was not affected by exposure to UV-A or visible light. The extract is most likely to have more than one mode of action, as it was active against three very different viruses (Sindbis is a membrane bound RNA virus, while poliovirus is a non-membrane bound RNA virus, and herpes simplex virus is a membrane bound DNA virus). The extract of *Carissa* was active at lower concentrations than any crude Nepalese plant extract yet tested in this ongoing screening, with antiviral activity present from 12 to 3 μg/mL. The extract was also very cytotoxic, with cytotoxic effects appearing at concentrations down to 25 μg/mL. *Carissa* species (including *C. carandas*) roots are known to be poisonous and contain cardiac glycosides (Joglekar and Gaitonde, 1970) which could be the source of the cytotoxicity. Cardiac glycosides are known to be slightly antiviral, but the concentrations of the pure substances tested were much higher than that of the crude *Carissa* extract (Hudson, 1990), indicating that another type of compound is the active ingredient.

The extract of *Streblus asper* also possessed significant antiviral activity. Interestingly, *S. asper*, like *C. carandas*, contains cardiac glycosides (Chatterjee et al., 1992). These cardinolides have been reported to be macrofilariacidal (active against the parasite, *Litomosoides carinii*, that causes filariasis) (Chatterjee et al., 1992), but no antiviral activity has been reported, nor have any constituents that are known to possess antiviral activity been identified from this source.

Bauhinia vahlii was active against herpes simplex virus at 25 μg/mL. The roots of *B. vahlii* are used by traditional healers in Nepal to treat cuts and wounds. The roots are also used to treat dysentery (Manandhar, 1990a, 1993),

and the seeds of *B. vahlii* are known to be used to treat boils (Bhattarai, 1993c; Manandhar, 1985). Another species of *Bauhinia*, *B. variegata* L., is used medicinally in Nepal to treat dysentery (Manandhar, 1993; Bhattarai, 1993b) and cuts and wounds (Manandhar, 1993). Perhaps the treatment of cuts and wounds also includes the treatment of other skin problems including herpes lesions and warts, both of which are caused by viruses. It is also possible that *B. vahlii* is only used to treat cuts and wounds (it did have significant antibacterial activity) and the antiviral activity is not known to the Nepalese people that were interviewed. *C. carandas*, like *B. vahlii*, is also used to treat dysentery, and also possesses antiviral activity, as do the extracts of *Mallotus philippensis*, *Streblus asper, Rumex hastatus* and *Terminalia alata,* all of which are used to treat dysentery. The link between the use of plants to treat dysentery and their antiviral activity would be interesting to pursue further, and perhaps could be the source of more antiviral extracts.

The therapeutic indices of many of the active extracts are not large (4-8, when known) but it must be noted that these are crude extracts, not purified compounds. It is possible that the compound responsible for the antiviral activity is not responsible for the cytotoxic effects of the extract, which would mean the therapeutic indices would become more desirable upon purification.

General Discussion

There have been many papers published in the last year on the subject of screening medicinal plants (or compounds from plants) for antiviral activity. The test viruses vary, but HIV (Abdel-Malek et al., 1996; Walder et al., 1995), and herpes simplex virus (Fukushima et al., 1995; Vlietinck et al., 1995; Cohen

et al., 1996) are popular. Abdel-Malek et al. have the interesting result that anti-HIV fractions from their test plants tend to be aqueous. Both McCutcheon et al. (1995) and Vlietinck et al. (1995) tested one hundred medicinal plants. Twelve out of the one hundred tested by McCutcheon et al. showed 'antiviral activity', while 27% of the plants tested by Vlietinck et al. were deemed to be antiviral (with the end point taken at 50% inhibition). Twelve percent of the plants were active against polio, and 8% against HSV.

Fourteen out of the twenty species tested from northern Nepal had at least partial antiviral activity (70%), with eight (40%) totally inhibiting at least one virus. Six of these species would not have been deemed to have any antiviral activity if they had not been exposed to some type of light treatment, and six had enhanced activity upon exposure to light. These figures are comparable with the southern Nepalese plants, of which sixteen of twenty-two (73%) were at least partially active and seven of twenty-two (32%) completely inhibited at least one virus. A smaller number of the southern species were photoactive: two were active only upon exposure to light, while the activity of two was enhanced.

The Asteraceae, Lamiaceae, and Hypericaceae are three families known to contain photosensitizers (Bohlman et al., 1973; Towers, 1984; Yip, 1993; Hudson et al., 1993b), and all three of these families were represented in the light activated extracts in this screen. The Asteraceae represents a ubiquitous world wide family, and species are present in several recent antiviral screenings (see Abdel-Malek et al., 1996; Vlietinck et al., 1995; and McCutcheon et al., 1995), but unfortunately light conditions are not mentioned or taken into account in these screens. Abdel-Malek et al. state in their discussion that they found high anti-HIV activity in the Asteraceae, but do not discuss the possibility of photosensitizers being responsible for this activity.

Hu and Hsiung (1989) have composed a detailed list of variables to take into consideration when screening for antiviral activity *in vitro*. Several of the factors worth mentioning are the host cell system, strain of virus used, the concentration of virus, the growth rate of the host cells, and the time between infection and observation of viral CPE.

All these factors were kept constant in my screen. The assays were read and results recorded as soon as the control wells, containing cells and virus, were completely infected with virus. The end point for my assays was taken as the minimum concentration of extract needed to inhibit 100% of the virus. Any extracts that were able to inhibit all the virus at the concentrations tested were classified as antiviral. This was a much more stringent criterion for antiviral activity than many of the other antiviral assays that have been published recently. Walder et al. (1995) present their results as 50% effective inhibitory dose in their anti-HIV assays, as do Vlietinck et al. (1995) in their screen against several viruses. Manolova et al. (1995) report their antiviral activity as a reduction of viral titres by 90, 95, 99 or 99.9%.

While this study did use 100% inactivation to define an antiviral extract, it is important to note that many extracts had partial antiviral activity. These extracts could contain compounds that are true antivirals but that were present at quantities insufficient to inactivate all infectious virus in the standard virus preparation (100 pfu/well), or compounds that slow the replication and/or spread of the virus.

Another factor which must be taken into account when discussing antiviral screenings is concentration of serum in the medium. Serum has been found to bind to antiviral components of plant extracts, inhibiting the activity. This is especially true when working with photosensitizers, such as α-terthienyl or hypericin (Hudson et al., 1994). Hudson et al. (1994) experimented with the

effect of serum on the anti-SINV activity of hypericin and α-terthienyl. Hypericin needed a minimal amount of serum to be active, but excess serum reduced activity. The relationship between serum concentration and activity was more simple for α-terthienyl, with maximum inhibition occurring at 0% serum, and activity decreasing with increasing serum concentration. To compensate for this fact, serum concentration in the test medium of my assays was set at 2%, while serum in the cell cultures not being used for testing was left at 10%.

The activity of *Carissa carandas* was thought interesting enough to pursue this extract further. The bioactivity guided fractionation can be seen in Chapter VI.

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Chapter V

Isolation and Identification of Several Bioactive Components from *Centipeda minima*

Introduction

In the antimicrobial screening of medicinal plants of Nepal, the crude methanolic extract of the whole plant of *Centipeda minima* (L.) A. Br. & Aschers (Asteraceae) was determined to be active against *Bacillus subtilis, Staphylococcus aureus, Mycobacterium phlei* and *Microsporum gypseum.* The activity against *Bacillus subtilis* was enhanced upon exposure to UV-A radiation. The zones of inhibition this extract displayed against these bacteria were quite large, and therefore easy to follow during bioactivity guided fractionation.

There have been no reports of *C. minima* containing known antimicrobial compounds. The plant has been documented as being used medicinally in Nepal to treat coughs and colds, headache (Manandhar, 1993) and blocked nose resulting from coughs and colds (Manandhar, 1994). The Nepali name for *C. minima* is 'Hachhyun', while the Tamang people call it 'Hachhi mran'. *C. minima* is also used throughout South East Asia to treat colds, nasal allergies and asthma (Wu et al., 1985). There have been several studies of *C. minima* based on the fact that it is used as an anti-allergy treatment. Wu and coworkers (1985, 1991) have shown that extracts of *C. minima* inhibit histamine release, with flavonoids, sesquiterpene lactones and an amide being the active components. The same research group also determined that sesquiterpenes were responsible for the activity of *C. minima* as a platelet activating factor (PAF) antagonist (Iwakami et al., 1992). Platelet

activating factor is released to induce platelet aggregation, and acts as a chemical mediator in anaphylaxis response (Iwakami et al., 1992).

In the NAPRALERT database (1993), the following compounds have been reported from *C. minima*: saponins, phenolic glycosides, stilbene derivatives, esters, sterols (Gupta and Singh, 1990; Bohlmann and Zhongliang, 1984), sesquiterpene lactones (Iwakami et al., 1992; Bohlmann and Zhongliang, 1984; Wu et al., 1985), a large number of volatile compounds and aurantiamide acetate (an amide) (Wu et al., 1985). There have also been several flavones, and diterpenic acids isolated from *Centipeda orbicularis* Lour (Bohlmann and Mahanta, 1979).

Methodology

Antibacterial Bioassays

All fractions obtained from separation procedures were assayed for antibacterial activity. Each fraction was dried under a stream of nitrogen gas and weighed, then redissolved in a suitable solvent at a concentration of 50 mg/mL. The disk assays were carried out as described in Chapter III.

Thin layer chromatography bioautography agar overlay was used to follow the bioactivity as fractionation occurred. The method used was from Saxena et al. (1995). Muller Hinton medium (BBL) was prepared with 0.6% Bacto agar (6 g/L) and 0.002% phenol red (0.002 g/L). The medium was autoclaved at 121 °C for 15 min and then maintained as a liquid at 45 °C in a water bath prior to use. Bacterial cultures were grown overnight in Muller Hinton medium and diluted to 10^6 cells/mL by serial dilution just before use.

The final concentration of bacteria in agar containing medium was 10^5 cells/mL.

In thin layer chromatography, silica gel G60 F_{254} alumina backed plates (8 x 8 cm) were used. Two identical chromatograms were run in appropriate solvent for each overlay. UV active compounds were detected at 254 and 366 nm on the reference chromatogram, which was then stained with vanillin:H_2SO_4 spray reagent. The other set was used for overlay.

The bacteria used for the TLC overlay were *Bacillus subtilis,* standard laboratory strain from The University of British Columbia microbiological collection, Department of Microbiology, and methicillin resistant *Staphylococcus aureus*, a gift from Dr. R. E. W. Hancock, Department of Microbiology, The University of British Columbia.

Minimum inhibitory concentrations of the purified compounds were determined by the following method, a modification of the method of Towers et al. (1985). A series of nine two-fold dilutions of the test compounds (dissolved in dimethylsulphoxide (DMSO) at a concentration of 25 mg/mL and diluted with Muller-Hinton broth (BBL) to a concentration of 2.5 mg/mL) and the control (gentamicin, 1 mg/mL in sterile water) were made with medium in a 96 well plate (Falcon 3072). Four replicates were performed, each one having 100 µL of serial dilution. One row was set up with medium only (control). A 18 h culture of the bacteria, grown in Muller-Hinton broth at 37 °C, was diluted to ~2 x 10^4 cells/mL in fresh medium. Aliquots (100 µL) of this were added to the wells of the 96 well plate. The optical density (O. D.) at 429 nm of each solution in a test well was read before incubation, and both 24 and 48 h later.

Plant Extract Preparation

Until light activity could be assessed, all procedures were carried out under low light conditions. Plant material was air dried and ground in a Wiley grinder with a 2 mm wire mesh. The powder (300 g) was exhaustively extracted with in 1,000 mL aliquots of MeOH over a period of several days. Each extraction took a minimum of 24 h. The sample was then suction filtered through Whatman # 1 filter paper, and washed with another 1,000 mL MeOH. The filtrate was evaporated to dryness under reduced pressure.

Chemical Separations

Liquid-Liquid Partition Chromatography

The crude methanolic extract was resuspended in aqueous methanol ($MeOH_{aq}$) ($MeOH:H_2O$, 3:2). This was then partitioned successively in order of increasing polarity with petroleum ether (pet. ether) and dichloromethane (CH_2Cl_2).

Column liquid chromatography

Separation of active fractions was tried using both silica (70-230 mesh) and Sephadex LH20 (Pharmacia) as the stationary phase, and a variety of suitable solvents.

Thin Layer Chromatography

Silica gel G60 F_{254} alumina backed plates were used with the appropriate solvent system. UV active compounds were detected at 254 and 366 nm, and then the TLC plate was stained with vanillin:H_2SO_4 spray reagent.

Vacuum Liquid Chromatography

TLC grade silica (Merck Kiesel gel 60) was used as the stationary phase, following a procedure modified from Pelletier et al. (1986). The solvent system used was petroleum ether with increasing amounts (0, 1, 2, 5, 10, 25, 50%) of dichloromethane, then dichloromethane with increasing amounts (0, 1, 2, 5, 10, 25%) of methanol. The column was finally washed with 50% MeOH in CH_2Cl_2, followed by 100% MeOH.

High Performance Liquid Chromatography (HPLC)

Active fractions were further separated on HPLC using a Waters 660E controller, and the Waters 994 programmable photodiode array (PDA) detector, with either a Waters RCm 8 x 10, or 25 x 10, NV C_{18} column. The UV detector was set to scan for absorption at 220 nm. The solvent conditions were isocratic with a ratio of 35:65 acetonitrile (MeCN) to water (H2O). Flow rate was 3 mL/min. for the 8 x 10 column, 20 mL/min. for the 25 x 10 column.

Structural Analysis

Ultraviolet Spectroscopy (UV-Vis)

Spectra from HPLC fractions were obtained during separation, on the Waters 994 PDA detector.

Mass Spectroscopy (MS)

EI obtained on a Kratos MS 50 mass spectrometer at 70 eV. All mass spectrometry was performed by the Mass Spectrometry Center at the Chemistry Department, University of British Columbia.

Nuclear Magnetic Resonance Spectroscopy (NMR)

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX-500 (500 MHz) instrument in $CDCl_3$, unless otherwise stated. The chemical shifts were recorded in parts per million (ppm) on a δ scale calibrated to $CDCl_3$ (7.24) as an internal standard.

Results

Bioactivity Guided Fractionation

Liquid-Liquid Partitioning

Partitioning the aqueous MeOH extract (51.5 g) with petroleum ether resulted in a fraction (7.5 g) that was only active against *B. subtilis* in the presence of UV-A radiation. The CH_2Cl_2 fraction (12.5 g) was active upon exposure to UV-A radiation and in the dark. These results are shown in Table 5.1, and Figure 5.1.

Table 5.1

Activities of *C. minima* liquid-liquid partition fractions against *Bacillus subtilis.*

Fraction	Presence of Zone of Inhibition in Dark	Presence of Zone of Inhibition UV-A Treatment
Petroleum Ether	NO	YES
Dichloromethane	YES	YES
Aqueous Methanol	NO	NO

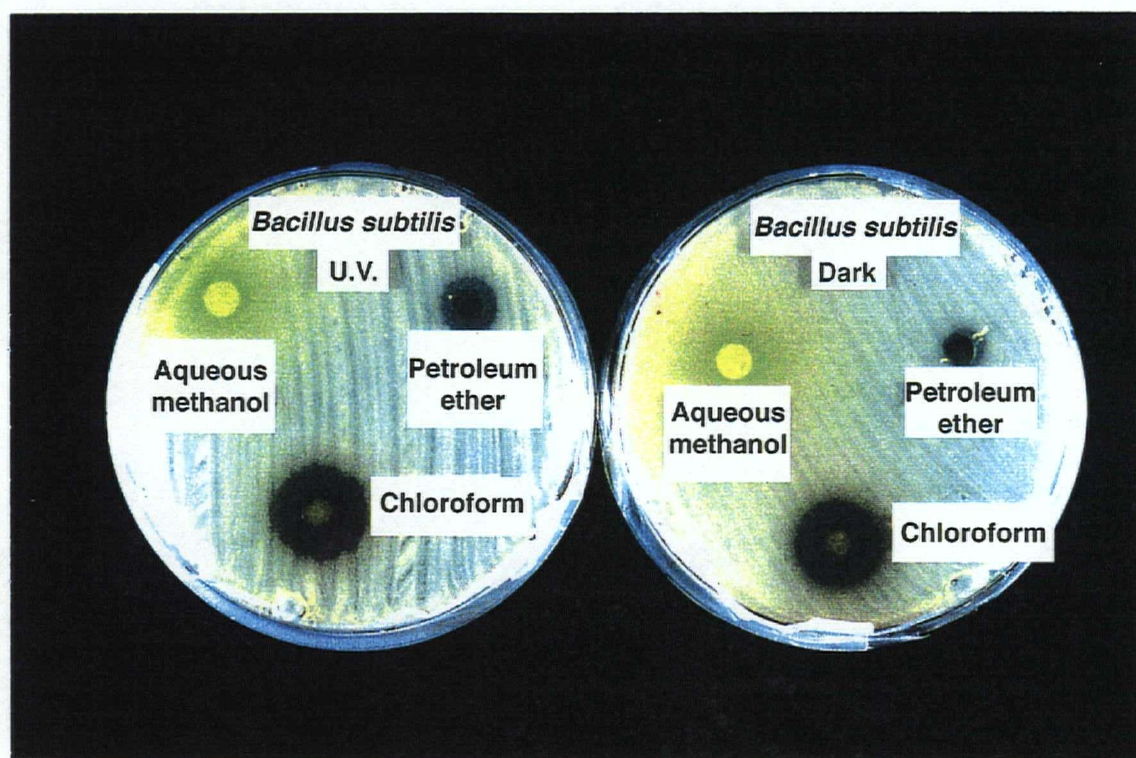


Figure 5.1. Activities of *C. minima* liquid-liquid partition fractions against *Bacillus subtilis* in dark and and UV-A radiation.

Fractionation Procedures

The active dichloromethane fraction was then put through further bioactivity guided fractionation. A silica (70-230) column (150 g) was run with 5 g of the active fraction. The solvent used was CH_2Cl_2, with increasing percentage of MeOH, starting at 1% and doubling. The resulting fractions (15 fractions of 500 mL each) were collected and tested by the TLC overlay method. The active fraction was eluted with 4% MeOH in CH_2Cl_2 (fraction 6). This fraction (782 mg) was run through another silica column (120 g) and 45 fractions of 20 mL each were collected. The activity was once again eluted at 4% MeOH in CH_2Cl_2 (fractions 4-10). These fractions were combined (41 mg) and run on a VLC (20 g), using benzene and ethyl acetate (EtOAc) as the solvent, in increasing increments similar to the silica columns. Twenty fractions of 20 mL were collected. The activity was located in fractions 9 and 10, which were combined and run on the preparative HPLC.

The resulting run on the HPLC (Waters RCm 8 x 10 NV C18 column) with an isocratic solvent ratio of 35:65 acetonitrile (MeCN) to water (H_2O) and an elution rate of 3 mL/min gave three bioactive peaks, **1** (retention time of 12.04 min, 8.6 mg), **2** (retention time of 13.43 min, 8.8 mg) and **3** (retention time of 17.64 min, 8.3 mg). The chromatogram can be seen in Figure 5.2.

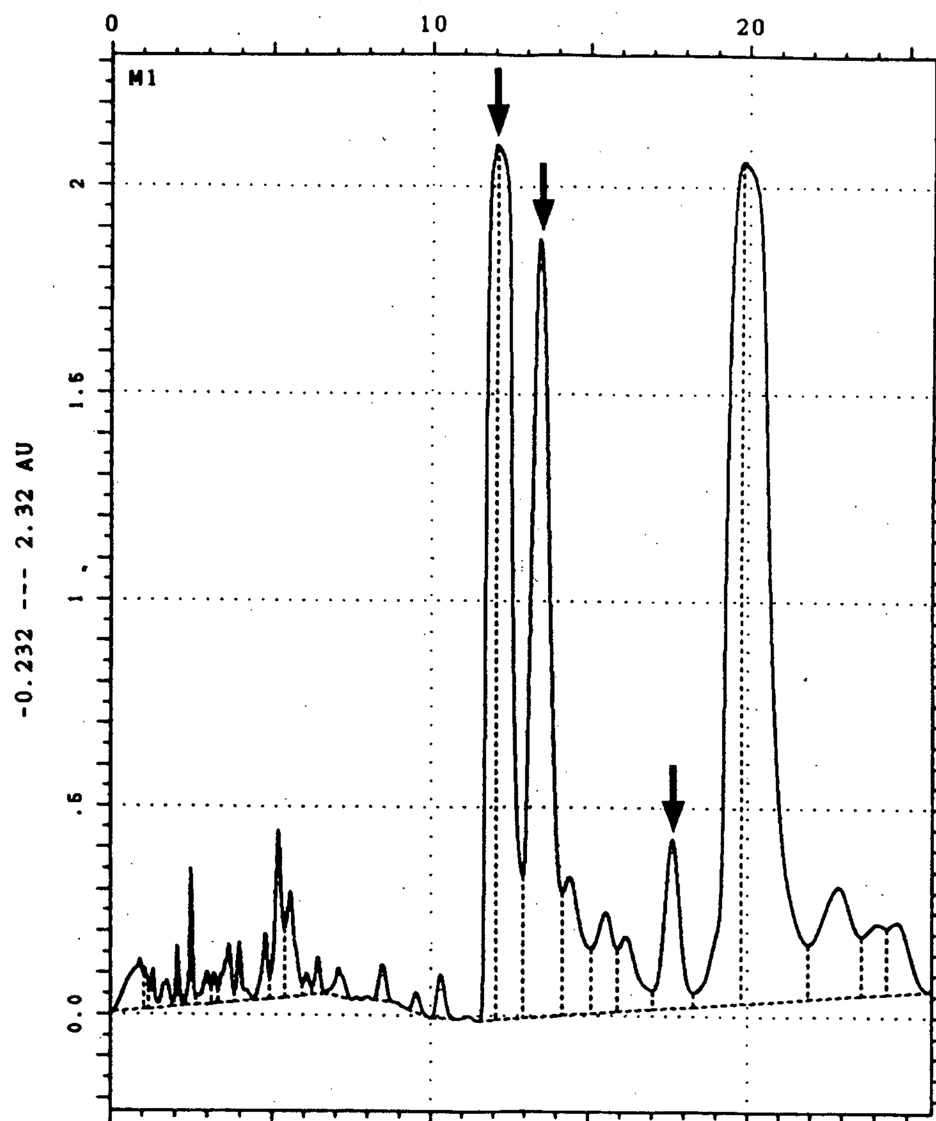


Figure 5.2. HPLC chromatogram of active components

(Isolated peaks indicated by arrows)

Structural Identification

Compound **1**:

The fraction containing compound **1** was found to be a 2:1 mixture of compound **1** and another compound whose NMR and MS spectra resembled compound **2**, and could not be separated further by HPLC, or TLC. Compound **1** was identified by MS and NMR as arnicolide D (α–methylacrylylplenolin, 6-O-methylacrylylplenolin), a sesquiterpene lactone of the plenolin type, and can be seen in Figure 5.3. From 300 g dried plant, 8.6 mg of the mixture was isolated. This corresponds to approximately 19 parts per million (ppm) of compound **1**.

Ultraviolet Spectroscopy

The UV spectrum of the mixture of compound **1** and compound **2** can be seen in Figure 5.4.

Mass Spectrometry

The EI spectrum of a solid probe sample with the relative abundance of fragments is shown in Figure 5.5

High Resolution Mass Spectrometery

The molecular ion mass of 332.16226 gave a molecular formula of $C_{19}H_{24}O_5$. The fragment analysis is shown in Appendix 1.

Proton Nuclear Magnetic Resonance Spectroscopy

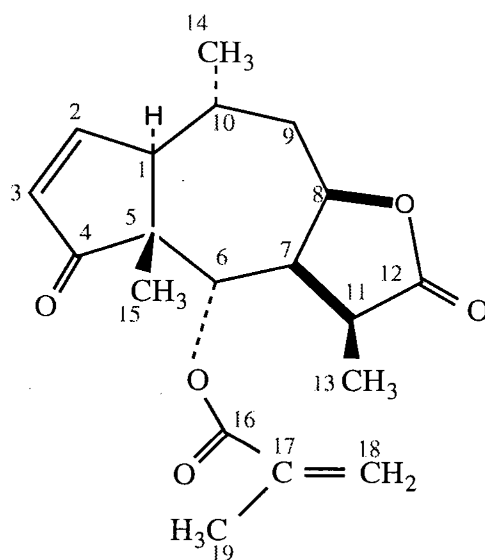
The ^{1}H-NMR spectrum of compound **1** can be seen in Figure 5.6. The assignments can be seen in Table 5.2.

Carbon Nuclear Magnetic Resonance Spectroscopy

The ^{13}C-NMR spectrum of compound **1** can be seen in Figure 5.7. The assignments are listed in Table 5.3.

HMQC

Proton carbon coupling from the HMQC experiment can be seen in Table 5.4.



1

Figure 5.3. Structure of 6-O-methylacrylylplenolin

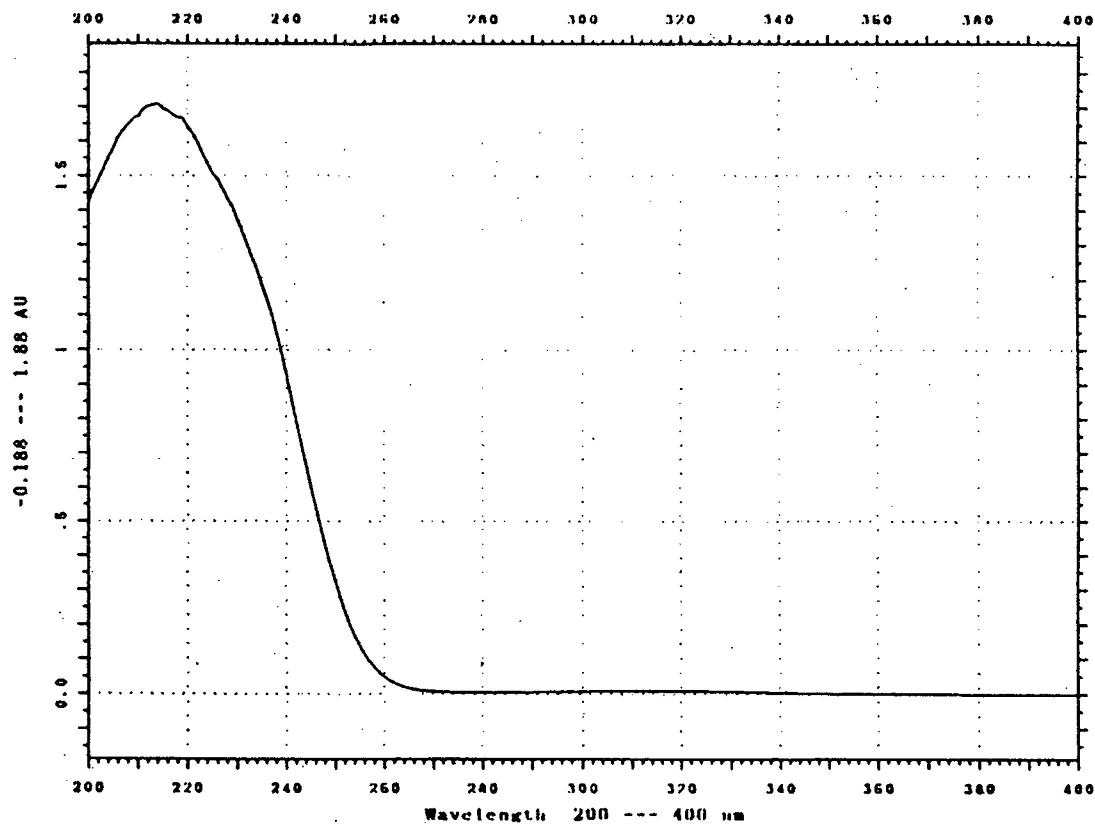


Figure 5.4. UV spectrum of mixture containing 6-O-methylacrylylplenolin

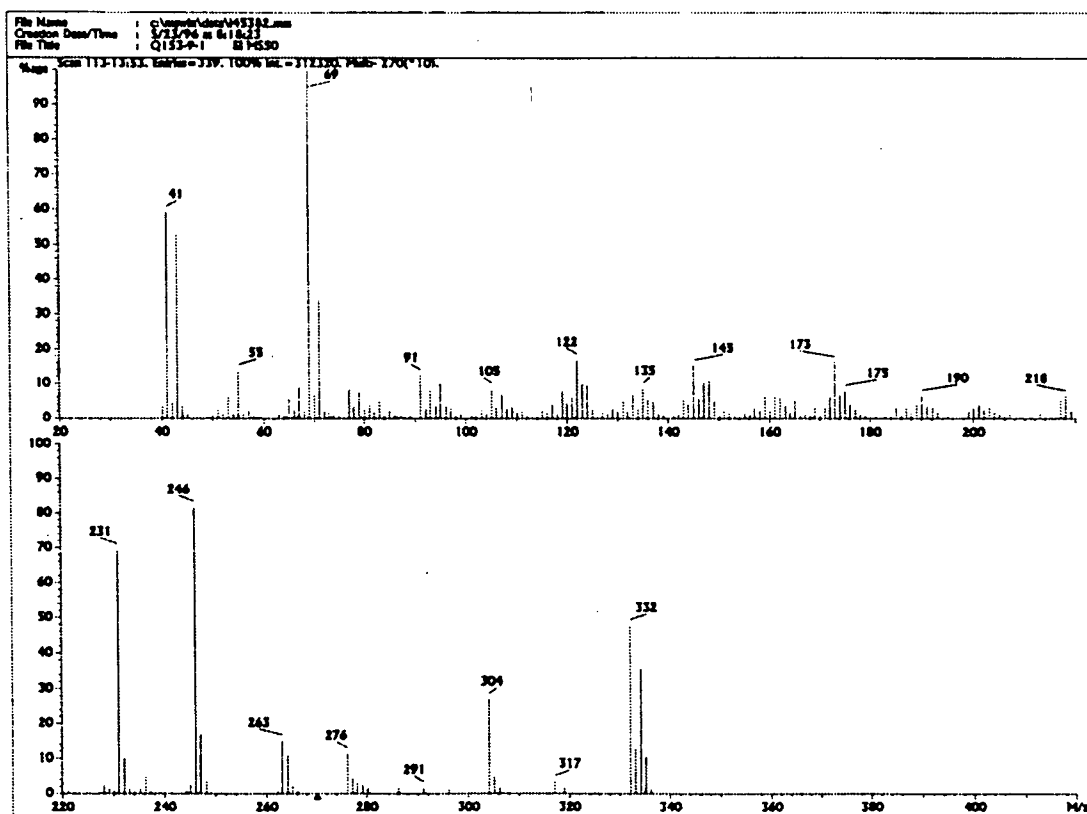


Figure 5.5. EI mass spectrum of mixture containing 6-O-methylacrylylplenolin

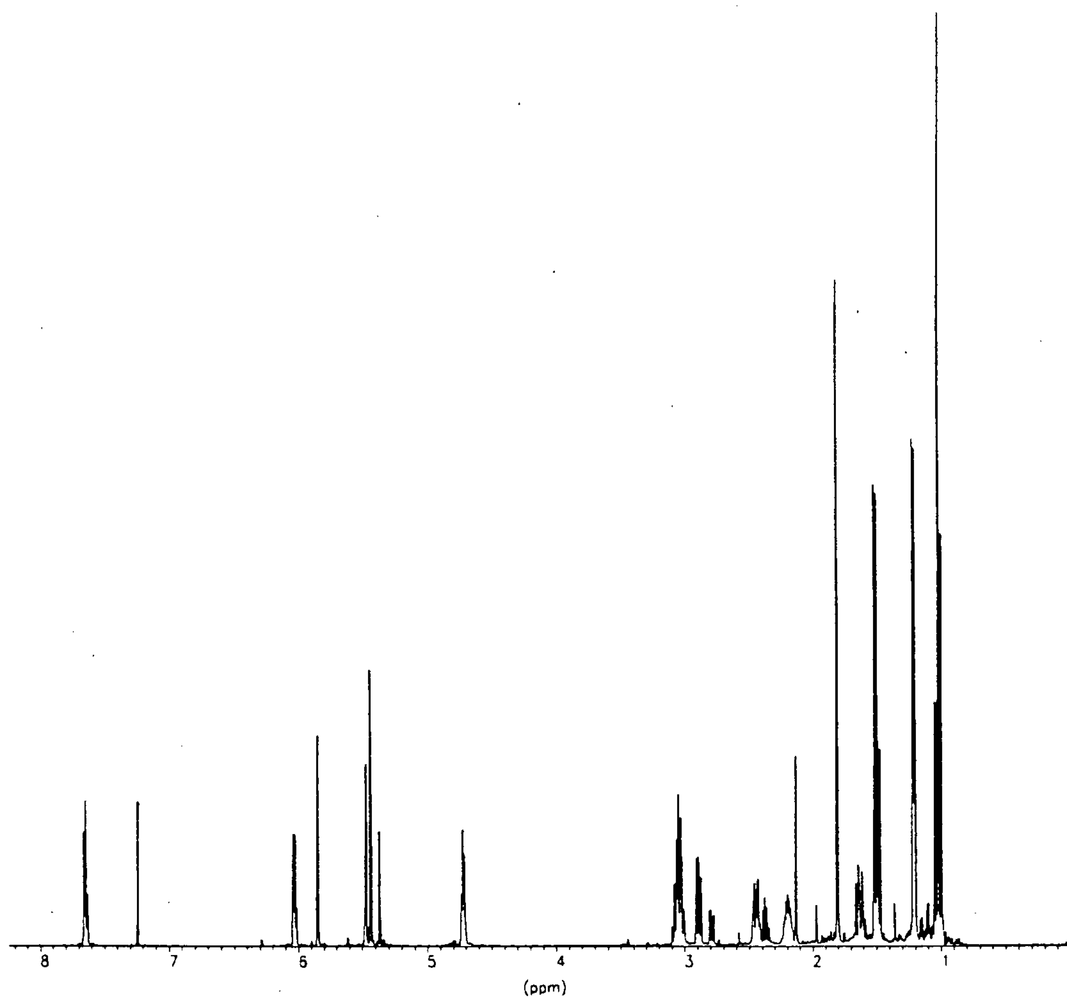


Figure 5.6. ^{1}H-NMR spectrum of mixture containing 6-O-methylacrylylplenolin

Table 5.2

^{1}H-NMR assignments for 6-O-methylacrylylplenolin

Chemical Shift	Position	Literature values (100 MHz, $CDCl_3$) (Poplawski et al., 1971)
1.03 (3H, s)	C15	1.06
1.21 (3H, d, J=6.67)	C14	1.24
1.51 (3H, d, J=7.44)	C13	1.54
1.63 (1H, ddd, J=1.91, 11.1, 15.3)	C9a	-
1.80 (3H, s)	C19	1.83
2.22 (1H, m)	C10	2.20
2.45 (1H, ddd, J=2.10, 5.72, 14.9)	C9b	2.48
2.89 (1H, dd, J=6.48, 10.3)	C7	2.9
3.07 (1H, m)	C1	3.07
3.08 (1H, m)	C11	3.07
4.73 (1H, dt, J=1.72, 6.10)	C8	4.75
5.44 (1H, br s)	C6	5.48
5.47 (1H, t, J=1.53)	C18a	5.50 (J=1.4)
5.85 (1H, s)	C18b	5.89 (J=1.5, 1)
6.03 (1H, dd, J=3.24, 6.29)	C3	6.06
7.65 (1H, dd, J=1.91, 6.30)	C2	7.68

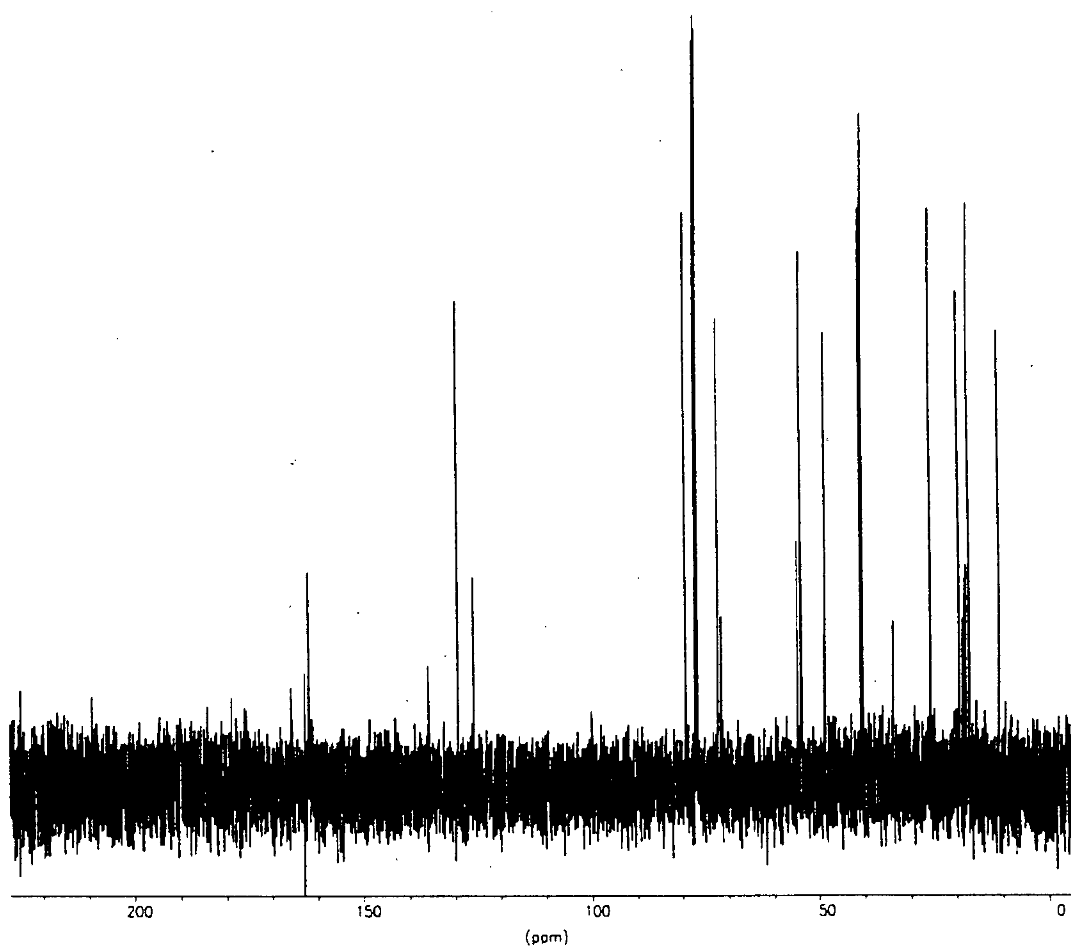


Figure 5.7. ^{13}C-NMR spectrum of mixture containing 6-O-methylacrylylplenolin

Table 5.3

^{13}C-NMR assignments for Compound 1

Chemical Shift	Carbon
209.320	C4
178.959	C12
175.584	C16
162.009	C2
129.448	C3
125.960	C18
79.440	C8
72.400	C6
54.904	C5
54.095	C1
48.685	C7
40.965	C9
40.450	C11
33.861	C17
25.812	C10
19.679	C14
18.116	C19
17.523	C15
10.894	C13

Table 5.4

HMQC data for Compound 1

Chemical Shift	Carbon	Proton Correlation
162.0	C2	7.65
129.4	C3	6.03
79.4	C8	4.73
72.4	C6	5.44
54.1	C1	3.07
48.7	C7	2.89
41.0	C9	2.45, 1.63
40.5	C11	3.08
25.8	C10	2.22
19.7	C14	1.21
18.2	C19	1.80
17.5	C15	1.03
10.9	C13	1.51

Compound **2**:

Compound **2** was identified to be arncolide C (6-O-isobutyroylplenolin), a sesquiterpene lactone of the plenolin type. The structure can be seen in Figure 5.8.

Ultraviolet spectroscopy

The UV spectrum of compound **2** is displayed in Figure 5.9.

Mass Spectrometry

The EI spectrum of a solid probe sample with the relative abundance of fragments is shown in Figure 5.10.

High Resolution Mass Spectrometery

The molecular ion mass of 334.17760 gave a molecular formula of $C_{19}H_{26}O_5$. The fragment analysis is shown in Appendix 1.

Proton Nuclear Magnetic Resonance Spectroscopy

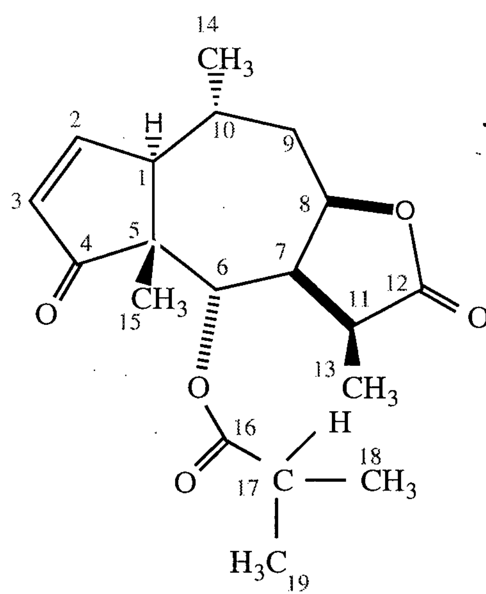
The ^{1}H-NMR spectrum of compound **2** can be seen in Figure 5.11. The assignments can be seen in Table 5.5.

Carbon Nuclear Magnetic Resonance Spectroscopy

The ^{13}C-NMR spectrum of compound **2** can be seen in Figure 5.12. The assignments are listed in Table 5.6.

HMQC

Proton carbon coupling from HMQC experiment can be seen in Table 5.7.



2

Figure 5.8. Structure of 6-O-isobutyroylplenolin

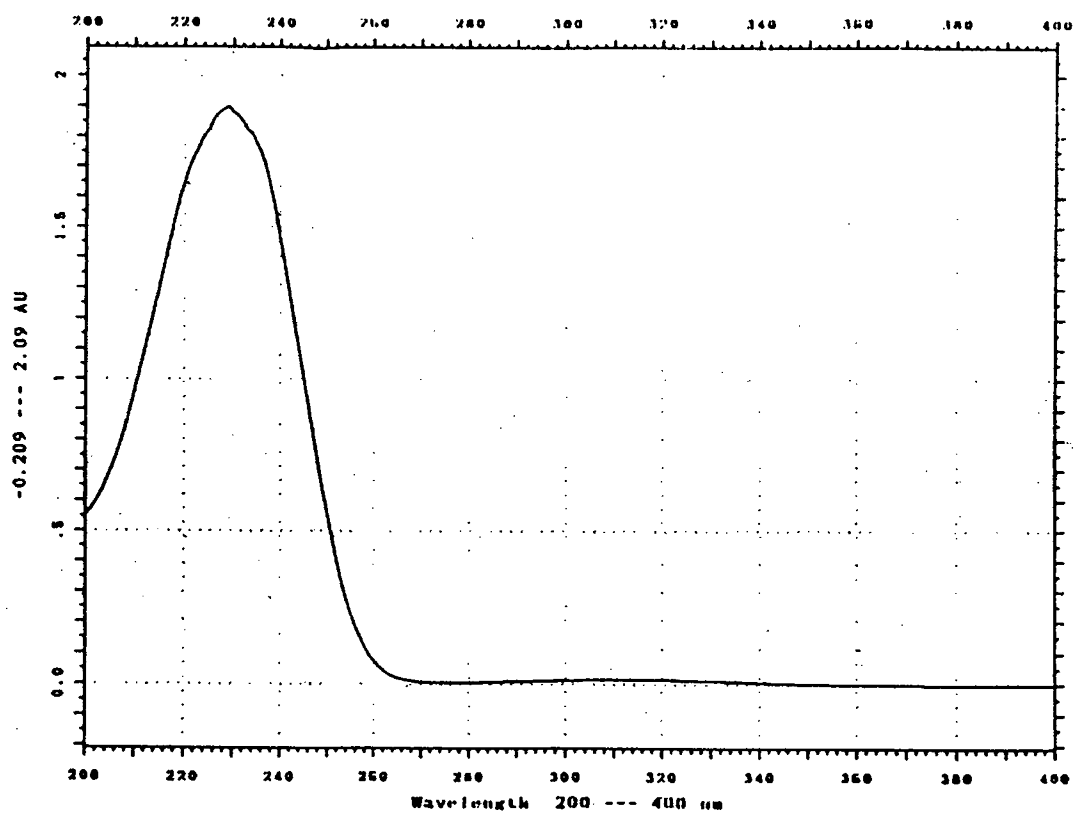


Figure 5.9. UV spectrum of 6-O-isobutyroylplenolin

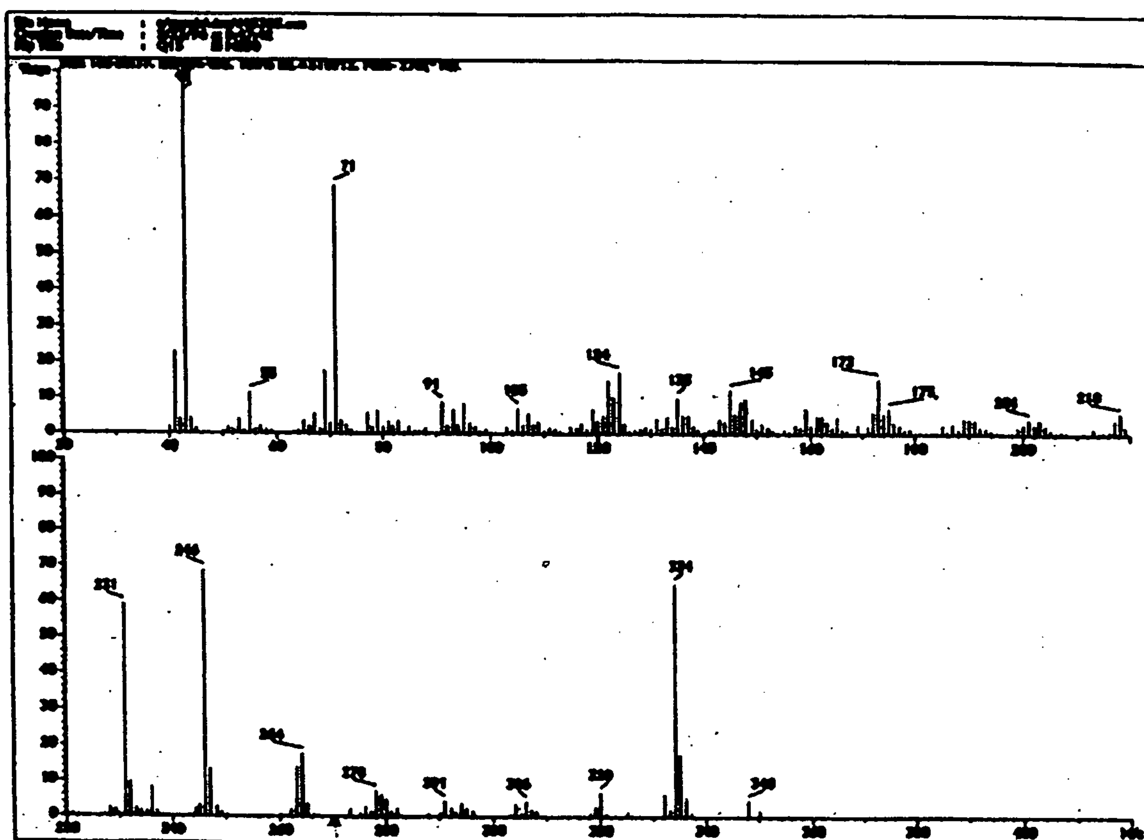


Figure 5.10. EI mass spectrum of 6-O-isobutyroylplenolin

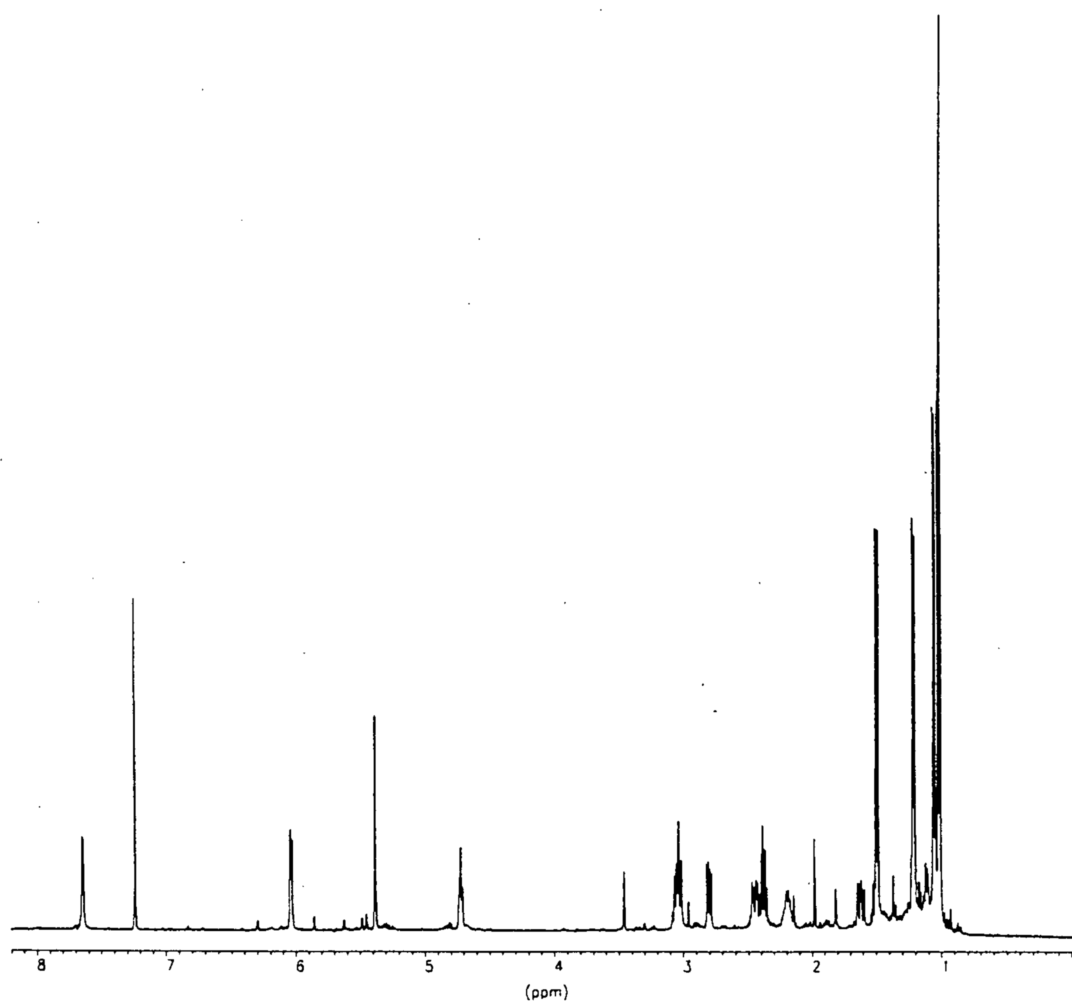


Figure 5.11. ^{1}H-NMR spectrum of 6-O-isobutyroylplenolin

Table 5.5

^{1}H-NMR assignments for 6-O-isobutyroylplenolin

Chemical Shift	Position	Literature value (400 MHz) (Wu et al., 1991)
1.01 (3H, s)	C15	1.03 (3H, s)
1.03 (3H, d, J=7.61)	C18 or C19	1.04 (3H, d, J=6.8)
1.06 (3H, d, J=7.61)	C18 or C19	1.08 (3H, d, J=6.6)
1.22 (3H, d, J=6.7)	C14	1.24 (3H, d, J=6.8)
1.50 (3H, d, J=7.43)	C13	1.51 (3H, d, J=7.6)
1.62 (1H, ddd, J=1.91, 11.1, 15.3)	C9a	1.65 (1H, ddd, J=21.7, 11.0, 15.4)
2.20 (1H, m)	C10	2.22 (1H, m)
2.39 (1H, sept., J=6.86)	C17	2.41 (1H, sept., J=7.1)
2.45 (1H, ddd, J=2.29, 6.10, 15.45)	C9b	2.48 (1H, ddd, J=2.2, 6.1, 15.4)
2.80 (1H, dd, J=6.49, 10.11)	C7	2.83 (1H, dd, J=6.4, 10.1)
3.00 (1H, m)	C1	3.05-3.12 (1H, m)
3.05 (1H, m)	C11	3.09 (1H, dq, J=10.3, 6.1)
4.73 (1H, dt, J=1.9, 6.1)	C8	4.75 (1H, ddd, J=1.7, 6.1, 6.4)
5.39 (1H, s)	C6	5.40 (1H, br s)
6.05 (1H, dd, J=3.24, 6.1)	C3	6.05 (1H, dd, J=3.2, 6.0)
7.64 (1H, dd, J=1.91, 6.1)	C2	7.68 (1H, dd, 1.9, 6.0)

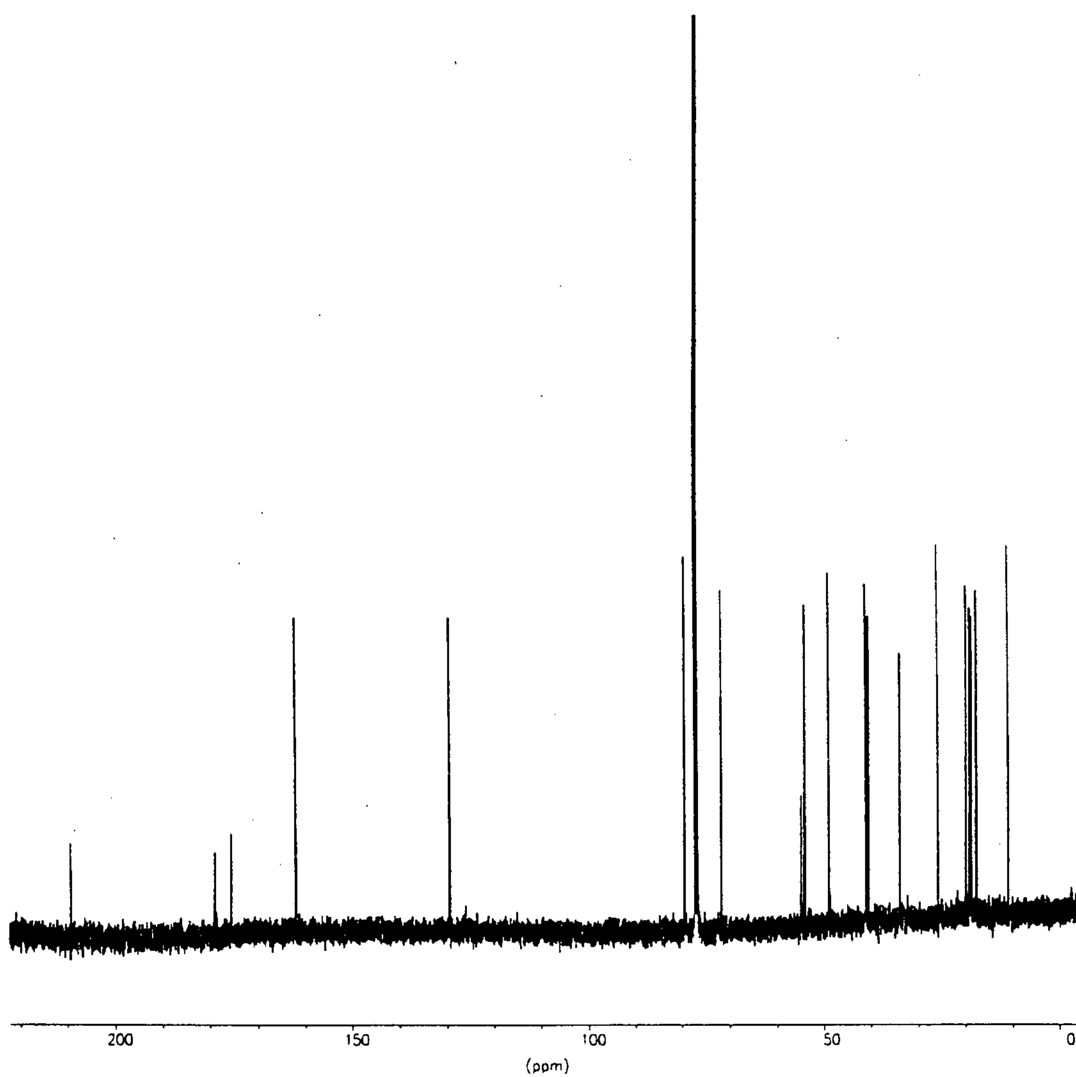


Figure 5.12. ^{13}C-NMR spectrum of 6-O-isobutyroylplenolin

Table 5.6

^{13}C-NMR assignments for 6-O-isobutyroylplenolin

Chemical Shift	Carbon
209.4	C4
179.0	C12
175.6	C16
162.0	C2
129.5	C3
79.4	C8
71.8	C6
54.5	C5
54.0	C1
49.9	C7
41.0	C9
40.5	C11
33.8	C17
25.8	C10
19.7	C14
18.9	C19 or C18
18.5	C18 or C19
17.5	C15
10.9	C13

Table 5.7

HMQC data for 6-O-isobutyroylplenolin

Chemical Shift	Carbon	Proton Correlation
162.0	C2	7.65
129.5	C3	6.05
79.4	C8	4.69
71.8	C6	5.38
54.0	C1	3.00
49.9	C7	2.79
41.0	C9	2.44, 1.61
40.5	C11	3.05
33.8	C17	2.38
25.8	C10	2.19
19.7	C14	1.21
18.9	C19 or C18	1.04
18.5	C18 or C19	1.08
17.5	C15	1.01
10.9	C13	1.49

Compound **3**:

Compound **3** was identified as 6-O-angeloylplenolin (brevifolin, brevilin-A), a sesquiterpene lactone of the plenolin type. The structure can be seen in Figure 5.13.

Ultraviolet spectroscopy

The UV spectrum of compound **3** can be seen in Figure 5.14.

Mass Spectrometry

The EI spectrum of a solid probe sample with the relative abundance of fragments is shown in Figure 5.15.

High Resolution Mass Spectrometery

The molecular ion mass of 346.17775 gave a molecular formula of $C_{20}H_{26}O_5$. The fragment analysis is shown in Appendix 1.

Proton Nuclear Magnetic Resonance Spectroscopy

The ^{1}H-NMR spectrum of compound **3** can be seen in Figure 5.16. The assignments can be seen in Table 5.8.

Carbon Nuclear Magnetic Resonance Spectroscopy

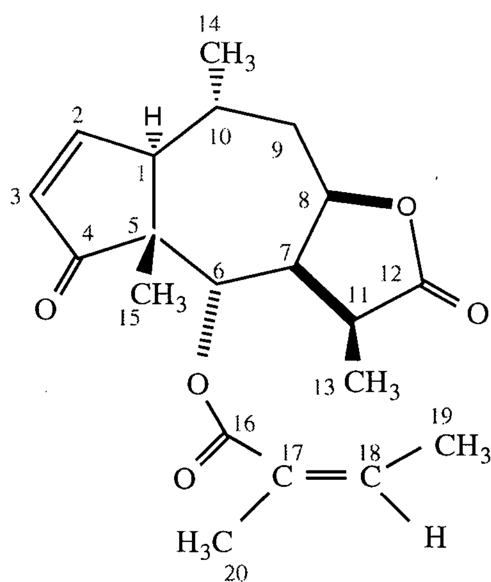
The ^{13}C-NMR spectrum of compound **3** can be seen in Figure 5.17. The assignments are listed in Table 5.9.

HMQC

Proton carbon coupling from HMQC experiment can be seen in Table 5.10.

HMBC

HMBC experimental results can be seen in Table 5.11



3

Figure 5.13. Structure of 6-O-angeloylplenolin

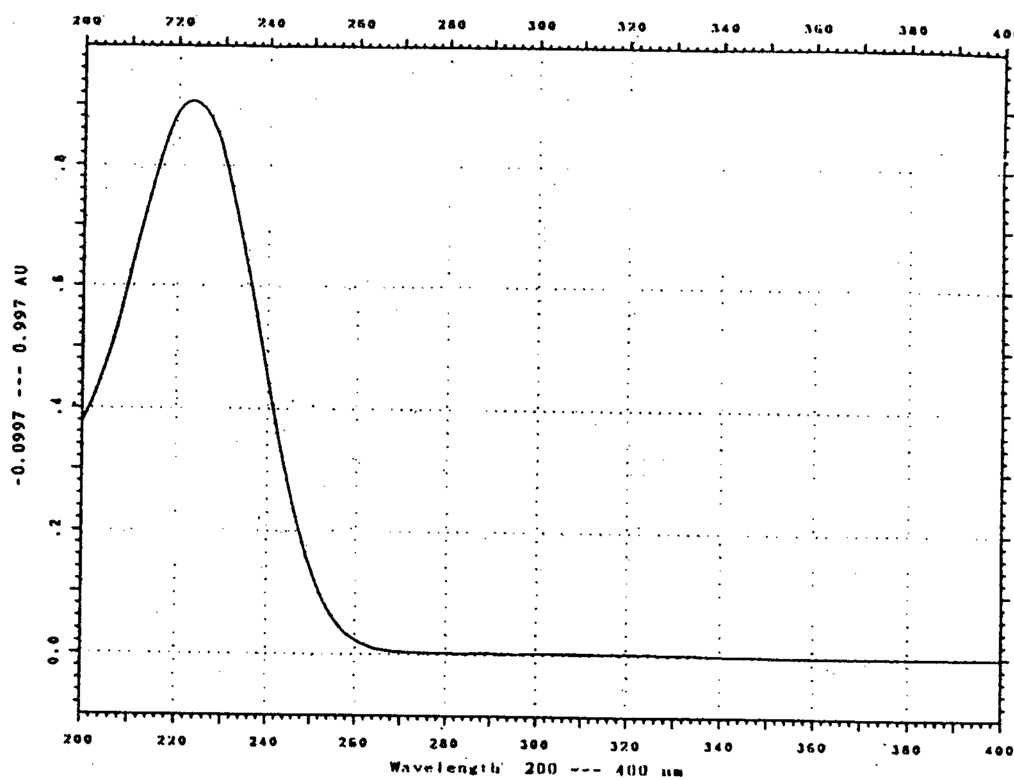


Figure 5.14. UV spectrum of 6-O-angeloylplenolin

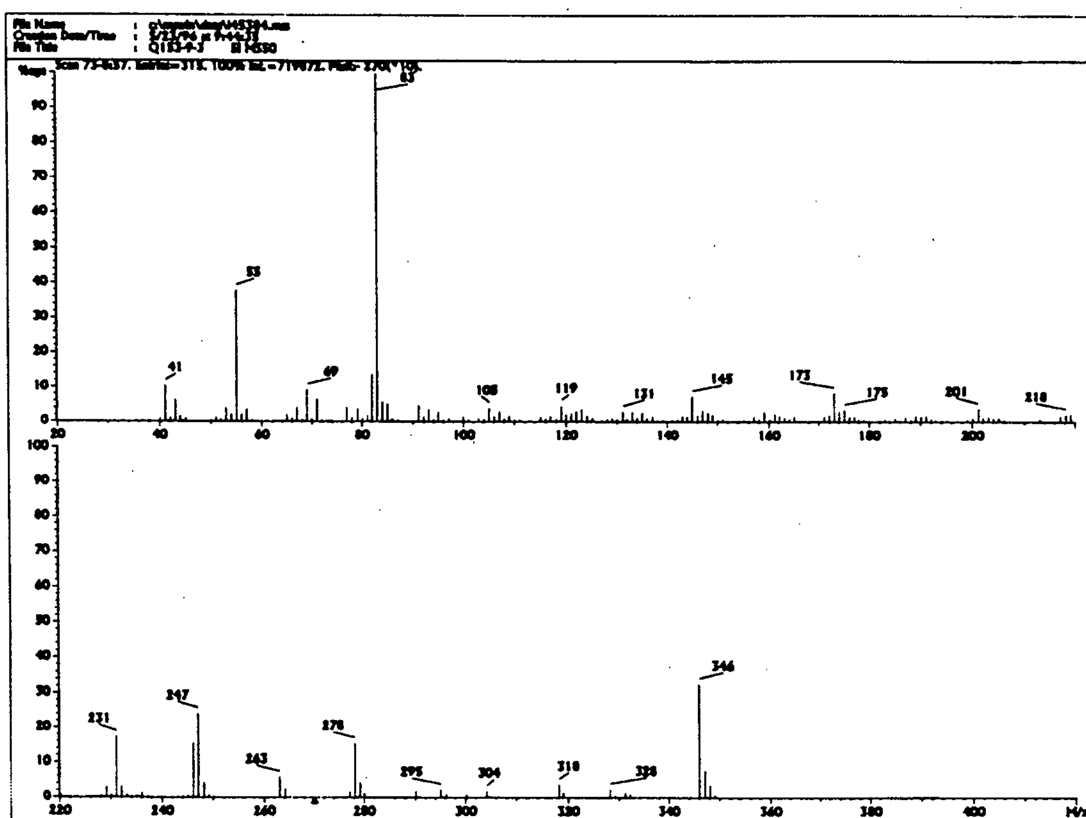


Figure 5.15. EI mass spectrum of 6-O-angeloylplenolin

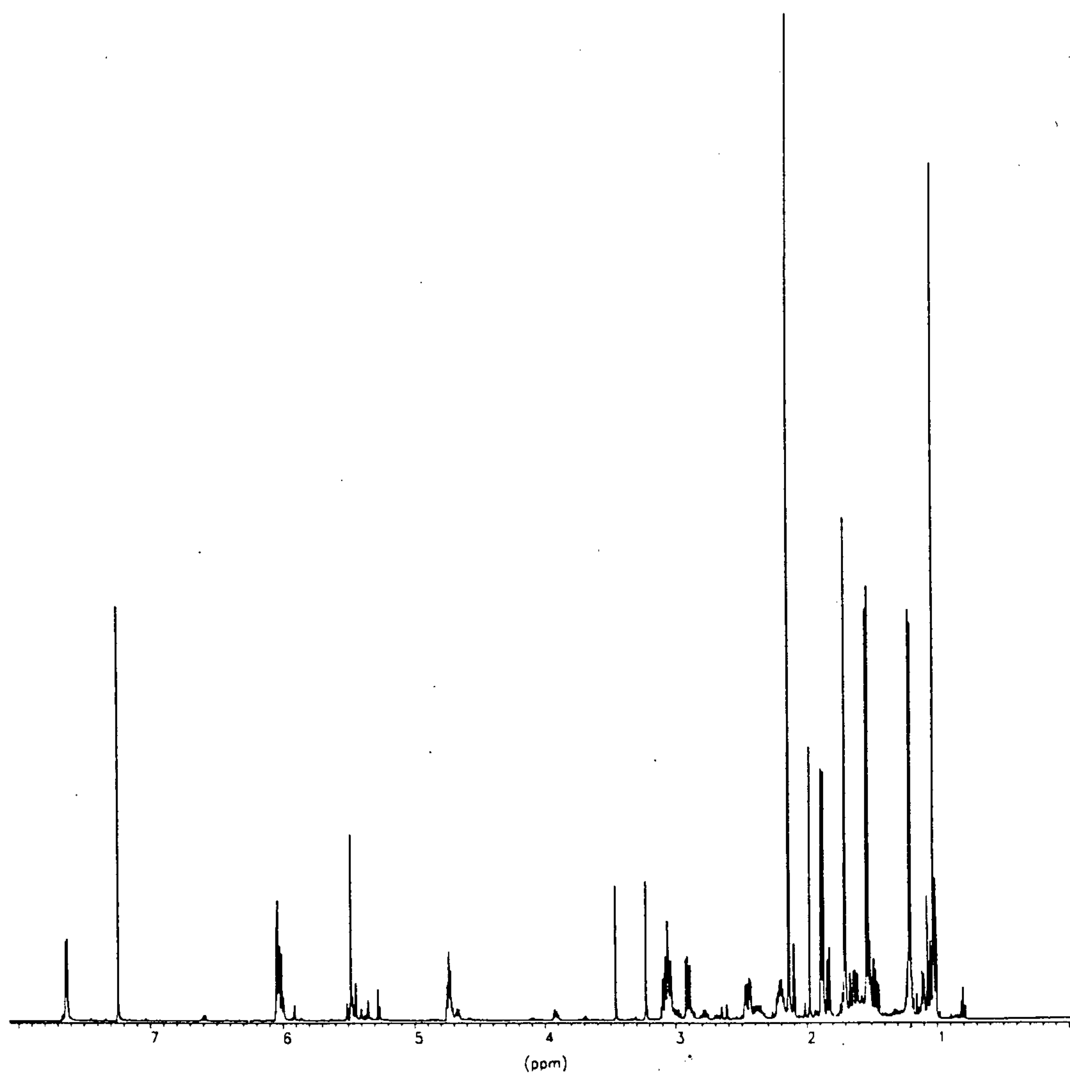


Figure 5.16. ^{1}H-NMR spectrum of 6-O-angeloylplenolin

Table 5.8

^{1}H-NMR assignments for 6-O-angeloylplenolin

Chemical Shift	Position	Literature Values (Itoigawa et al., 1981) (100 MHz, $CDCl_3$)
1.03 (3H, s)	C15	1.06 (3H, s)
1.21 (3H, d, J=6.68)	C14	1.23 (3H, d, J=7.0)
1.53 (3H, d, J=7.44)	C13	1.56 (3H, d, J=7.0)
1.63 (1H, ddd, J=1.91, 11.1, 15.3)	C9a	
1.70 (3H, dq J=1.52)	C20	1.75 (3H, br. s)
1.88 (3H, dq, J=1.52, 7.24)	C19	1.93 (3H, br. d, J=8.0)
2.2 (1H, m)	C10	
2.45 (1H, ddd, J=2.29, 5.91, 15.26)	C9b	
2.91 (1H, dd, J=6.68, 10.3)	C7	
3.05 (1H, m)	C1	
3.06 (1H, m)	C11	
4.73 (1H, dt, J=1.72, 6.1)	C8	4.77 (1H, m)
5.48 (1H, s)	C6	5.50 (1H, br. s)
6.01 (1H, qq, J=1.33, 7.24)	C18	6.04 (1H, m)
6.03 (1H, dd, J=3.05, 6.1)	C3	6.05 (1H, dd, J=3.0, 6.0)
7.63 (1H, dd, J=1.91, 6.1)	C2	7.67 (1H, dd, J=2.0, 6.0)

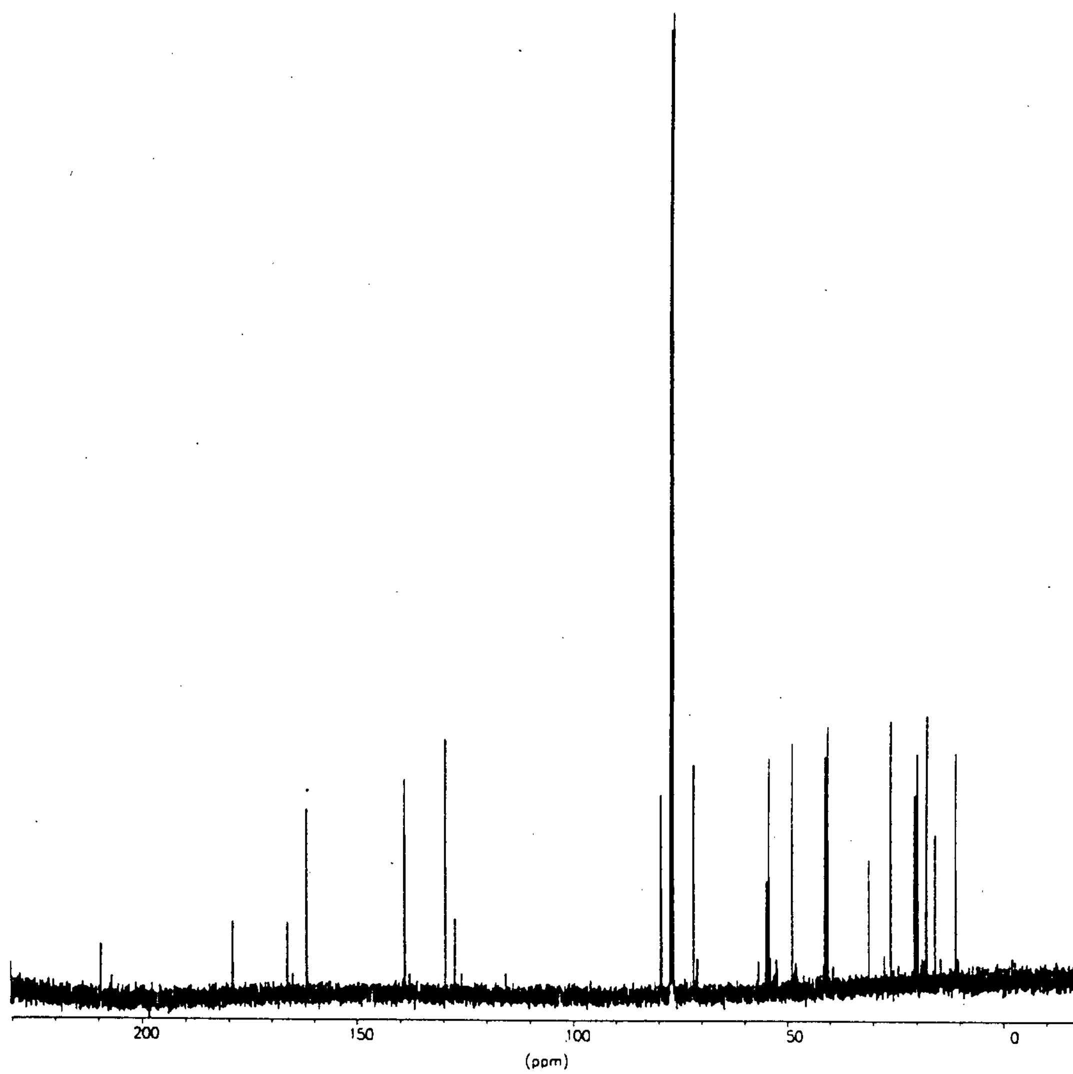


Figure 5.17. ^{13}C**-NMR spectrum of 6-O-angeloylplenolin**

Table 5.9

^{13}C-NMR assignments for 6-O-angeloylplenolin

Chemical Shift	Carbon	Literature Values (Itoigawa et al., 1981) (100 MHz, $CDCl_3$)
209.5	C4	208.9
179.0	C12	178.5
166.3	C16	165.9
161.8	C2	161.4
139.0	C18	138.6
129.5	C3	129.3
127.3	C17	127.0
79.5	C8	79.3
71.9	C6	71.7
54.9	C5	54.8
54.3	C1	54.2
48.9	C7	48.8
41.0	C9	41.0
40.5	C11	40.4
25.8	C10	25.8
20.4	C20	20.4
19.7	C14	19.7
17.6	C15	17.6
15.7	C19	15.6
11.0	C13	11.0

Table 5.10

HMQC data for 6-O-angeloylplenolin

Chemical Shift	Carbon	Proton Correlation
161.8	C2	7.63
139.0	C18	6.01
129.5	C3	6.03
79.5	C8	4.73
71.9	C6	5.48
54.3	C1	3.05
48.9	C7	2.91
41.0	C9	2.45, 1.63
40.5	C11	3.06
25.8	C10	2.2
20.4	C20	1.69
19.7	C14	1.21
17.6	C15	1.03
15.7	C19	1.88
11.0	C13	1.53

Table 5.11

HMBC data for 6-O-angeloylplenolin

^{1}H Chemical Shift	Carbon Attachment	Coupled to
1.03	C15 (17.6)	209.5 (C4), 71.9 (C6), 54.9 (C5)
1.21	C14 (19.7)	54.9 (C5), 41.0 (C9), 25.8 (C10),
1.53	C13 (11.0)	179.0 (C12), 48.9 (C7), 40.5 (C11),
1.63	C9 (41.0)	25.8 (C10)
1.70	C20 (20.4)	166.3 (C16), 139.0 (C18), 127.3 (C17)
1.88	C19 (15.8)	139.0 (C18), 127.3 (C17), 20.4 (C20)
2.20	C10 (25.8)	79.5 (C8)
2.45	C9 (41.0)	79.5 (C8), 48.9 (C7), 25.8 (C10), 19.7 (C14)
2.91	C7 (48.9)	179.0 (C12), 71.9 (C6), 54.9 (C5), 40.5 (C11)
3.05	C1 (54.3)	161.8 (C2), 129.5 (C3), 54.9 (C5), 41.0 (C9), 25.8 (C10), 17.6 (C15)
3.06	C11 (40.5)	179.0 (C12), 71.9 (C6), 48.9 (C7), 11.0 (C13)
4.73	C8 (79.5)	71.9 (C6), 25.8 (C10)
5.48	C6 (71.9)	209.5 (C4), 166.3 (C16), 129.4 (C3), 79.5 (C8), 54.9 (C5), 48.9 (C7), 40.5 (C11)
6.01	C18 (139.0)	20.4 (C20)
6.03	C3 (129.5)	54.9 (C5)
7.63	C2 (161.8)	209.5 (C4), 129.5 (C3), 54.9 (C5)

Antibacterial activity

The antibacterial activities of the three sesquiterpene lactones are shown in Table 5.12.

Table 5.12

Minimum inhibitory concentrations[a] of the isolated sesquiterpene lactones

(in μg/mL) (no light treatment)

Compound	*Bacillus subtilis*	*Staphylococcus aureus* (methicillin resistant)	*Staphylococcus aureus* (methicillin sensitive)
1	150	300	75
2	150	300	38
3	300	600	75
gentamicin	38	not active	9

[a]Minimum inhibitory concentrations: the minimum concentration of test compound needed to completely inhibit the growth of the bacteria.

Discussion

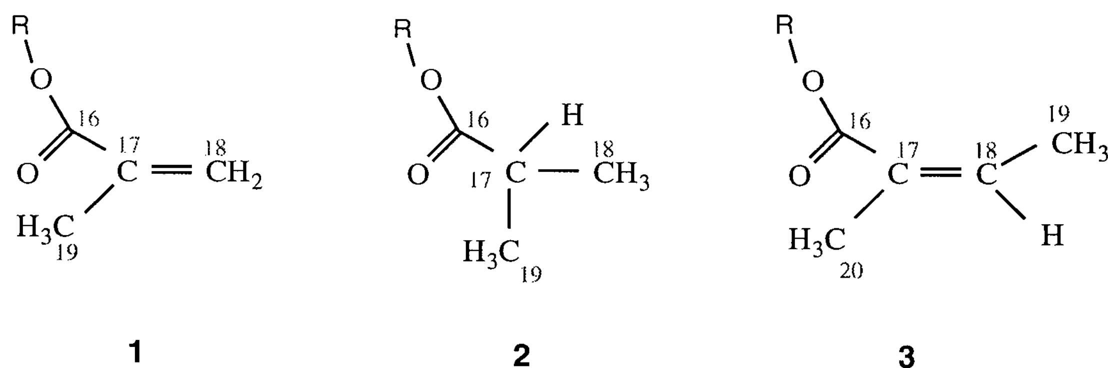
The light activated petroleum ether fraction was not pursued as a lead for antimicrobial compounds as species of the Asteraceae are known to contain photoactive polyynes. These compounds are known to be light active against bacteria and fungi, and are soluble in petroleum ether. Because they are unstable it was decided that the dichloromethane fraction would make a better choice for bioactivity guided fractionation.

The mixture of compounds **1** and **2** could not be further separated by HPLC or TLC. Activities reported for compound **1** are actually the activities for a 2:1 mixture of compounds **1** and a compound that has NMR and MS data similar to that of compound **2**. Since compound **2** was identified first, the proton and carbon NMR peaks of the compound resembling compound **2** in the mixture could easily be identified, leaving the remaining peaks of compound **1** to be analyzed.

All of the compounds were determined to be plenolin (13β-11, 13-dihydrohelenalin) esters. The structures of both plenolin and helanin can be seen in Figure 5.18.

Compound **1**, 6-O-methacrylylplenolin, α–methylacrylylplenolin, or Arnicolide D, has not previously been isloated from *Centipeda*, but it has been isolated from species of *Arnica*, another genus in the Asteraceae. Poplawski et al. (1971). isloated it from *Arnica montana* L., along with compound **2**. Herz and Sosa (1988) isolated compound **1** from *Arnica acaulis* (Walt.) B.S.P., together with compounds **2** and **3**. The molecular ion mass of compound **1**, 332.16226, gave a molecular formula of $C_{19}H_{24}O_5$. This fit the molecular formula of 6-O-methacrylylplenolin. The proton NMR spectrum compares well to that reported by Poplawski et al., although the comparison was made only

after the structure had been finalized by HMQC. Both the proton and carbon NMR spectra of compound **1** are very similar to that of compound **2**. The major differences in the proton spectra of compounds **1** and **2** are the side chain protons. The signals of the methylene protons of C18 can be seen at 5.47 and 5.85 ppm in the spectrum of compound **1** (Figure 5.6).



The C19 methyl protons are a singlet at 1.80 ppm. As C17 is quaternary in compound **1**, the proton signal that is present in the spectrum of compound **2** (Figure 5.11) at 2.39 ppm, is absent. Looking at the carbon spectrum, the signal for C18 of compound **1** can be seen at ~126 ppm (Figure 5.7), while in compound **2** it is at ~19 ppm (Figure 5.12). This is because the bond between C18 and C17 in compound **1** is sp^2, while in compound **2** it is sp^3. Compound **1** has not previously been reported to have antibacterial activity, although sesquiterpene lactones are known to be biologically active, as will be discussed later.

Compound **2,** arncolide C, or isobutyroylplenolin, had been previously isolated from *C. minima* by Bohlmann and Zhongliang (1984). It was previously known from *Arnica montana* (Poplawski et al., 1971). Arncolide C

was reisolated from *C. minima* by Wu et al. (1985) in a bioactivity guided fractionation looking for compounds showing significant antiallergy activity. While plenolin type sesquiterpene lactones are known to possess antibacterial activity, compound **2** has not been tested previous to my investigation. Compound **2** was determined to be arncolide C based on MS and NMR data. The molecular ion peak of 334.17760 gave a molecular formula of $C_{19}H_{26}O_5$, which has two protons more than compound **1**. This, together with comparison of NMR data with the literature indicated that compound **2** was arncolide C. The assignments of the chemical shifts in both the carbon and proton spectra were confirmed by HMQC results.

Compound **3** was identified to be 6-O-angeloylplenolin, brevilin-A, or brevifolin. It had been previously isolated from *C. minima* by Bohlmann and Zhongliang (1984). It was previously known from *Helenium brevifolium* (Herz et al., 1959), *H. alterniflolium* (Herz et al., 1968), and *H. autumnale* L. (Itoigawa et al., 1981). 6-O-Angeloylplenolin was reisolated from *C. minima* by Iwakami et al. (1992) in bioactivity guided fractionation research looking for compounds showing inhibitory activity on the binding of platelet activating factor (PAF) to rabbit platelets, a bioassay used to determine antiallergy compounds.

The structure of compound **3** was assigned based on high resolution MS and NMR data. The molecular ion peak of 346.17775 gave a molecular formula of $C_{20}H_{26}O_5$. This corresponded to a compound having the same basic structure as compound **2**, but with an extra carbon. The ^{13}C NMR spectrum confirmed this, with twenty carbon peaks present. The ^{13}C NMR spectrum of compound **3** differed from that of compound **2** in the fact that the carbonyl signal of C16 had shifted from 175.6 to 166.3 ppm, and the methyl signals at 18.5 and 18.9 ppm (C18 and 19) were not present. A new methyl peak at 20.4 (C20) was present, as well as signals at 139 and 33.8 ppm. These were assigned as C18

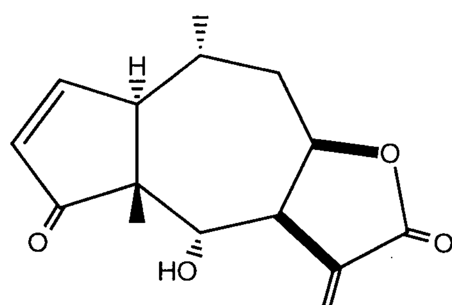
and C17 respectively. The proton and HMBC and HMQC spectra confirmed the structure of compound **3** as 6-O-angeloylplenolin.

Sesquiterpene lactones possessing cytotoxic or antimicrobial activity number approximately 150 (Bérdy et al., 1982). The compounds which I have isolated from *C. minima* belong to the largest class of sesquiterpene lactones, the pseudoguaianolides (Yoshioka et al., 1973). Several pseudoguaianolides, such as helenalin and plenolin (see Figure 5.21), have significant antibacterial activity. Both these compounds have an MIC of 100 μg/mL against both *Bacillis subtilis* and *Staphylococcus aureus* (Lee et al., 1977). Compounds **1**, and **2** show similar activities, with MIC values against *B. subtilis* of 150 μg/mL. Compound **3** was less active than the parent plenolin structure, with an MIC of 300 μg/mL.

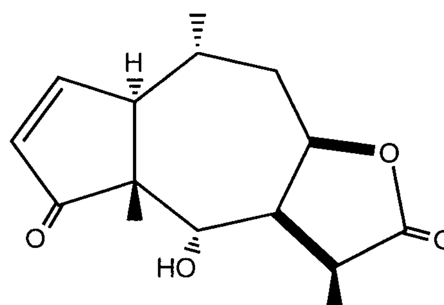
The antimicrobial activity of sesquiterpene lactones seems depend on the presence of a *beta* unsubstituted cyclopentenone ring moiety. Lee et al. (1977) determined that the saturated compound corresponding to helenalin gave at least a 10-fold decrease in antibacterial activity. This antibacterial activity appears to be independant of the presence or absence of an α-methylene-γ-lactone or α-methyl-γ-lactone moiety. This is demonstrated by the fact that both helenalin and plenolin have the same activity against both *B. subtilis* and *S. aureus* (Lee et al., 1977). However, this α-methylene-γ-lactone moiety is needed for significant cytotoxic activity (Bérdy et al., 1982), and antitumor activity (Rodriguez et al., 1976).

All three of the isolated compounds were determined to be bactericidal, not merely bacteriostatic. This was determined by trying to culture the media from the 96 well trays after 48 h incubation. If the compounds were merely bacteriostatic, the bacteria would resume growth after being removed from the test compound. This was not the case. It would also be interesting to test these

compounds for antileukemia activity, as the crude extract of *C. minima* was quite active (1mg/mL extract gave 66.2% inhibition against Jurkat (clone e6-1) cells) (Taylor et al., unpublished).



Helenalin



Plenolin

Figure 5.18. Structures of helenalin and plenolin

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Chapter VI

Isolation of an Antiviral Constituent of *Carissa carandas*

Introduction

In the course of the antiviral screening of medicinal plants of Nepal, the crude methanolic extract of the roots of*Carissa carandas* L. (Apocynaceae) was found to be particularly interesting. It was active against Sindbis virus (SINV) at 3 μg/mL, poliovirus (POLIO) at 6 μg/mL with partial activity at 3 μg/mL, and herpes simplex virus (HSV) at 12 μg/mL with partial activity at 6 μg/mL. These activities were not mediated by light.

Carissa carandas is an erect and spinous shrub which may reach up to 4 m in hight. It bears white, fragrant flowers, and berries which are purple when ripe (Manandhar, 1989b). It is used in Nepal to treat a plethora of ailments. Root juice is used to treat diarrhoea and dysentery, and is also dropped inside wounds (of animals) to remove worms or germs and helps to accelerate healing (Manandhar, 1995a). A root decoction or root paste is given to treat dysentery in cattle (Manandhar, 1989d, 1985), and root powder is said to eliminate lice (Manandhar, 1985). A decoction of young leaves is given to treat intermittent fever (Manandhar, 1986b). The ripe fruits are eaten to treat diarrhoea (Manandhar, 1989b), and the Tharu people, in the Bahraich District of Uttar Pradesh, India (on the Indo-Nepalese border), use the fruits of *C. carandas* to treat diarrhoea in animals (Singh et al., 1996). The Tharu name for *C. carandas* is 'karondath'.

Carissa congesta Wt. is used in India to treat measles and smallpox. The bark, along with the barks of *Combretum ovafolium* Roxb. (Combretaceae) and *Butea monosperma* (Lamk.) Taub. (Fabaceae) are powdered, but Sabnis and

Bedi neglect to mention how it is used in the treatment (Sabnis and Bedi, 1983). This is the only ethnobotanical reference to a species of *Carissa* being used to treat a disease known to be caused by a virus.

In the NAPRALERT database (1993), the following compounds have been reported from species of *Carissa*: sterols, diterpene alcohols, ursolic acid, methyl ursolate (Pakrashi et al., 1968), cardenolidescarissone, carindone (Singh and Rastogi, 1972), lignans (Pal et al., 1975; Achenbach et al., 1983), pentacyclic triterpenoids, aromatic compounds (Achenbach et al., 1983), sesquiterpenes (Achenbach et al., 1983, 1985), and 2-hydroxyacetophenone (Achenbach et al., 1983; Bentley and Brackett, 1984).

Methodology

Antiviral Bioassays

All fractions obtained from separation procedures were assayed for antiviral activity against HSV. The sample was dried under a stream of nitrogen gas and weighed, then redissolved in either MeOH or DMSO at a concentration of 40 mg/mL. The antiviral assays were carried out as described in Chapter IV.

Plant Extract Preparation

Until light activity could be assessed, all procedures were carried out under low light conditions. Plant material was air dried and ground in a Wiley grinder with a 2 mm wire mesh. The powder (700 g) was exhaustively extracted with in 1,000 mL aliquots of MeOH over a period of several days. Each extraction took a minimum of 24 h. The sample was then suction filtered through Whatman # 1 filter paper, and washed with another 1,000 mL MeOH. The filtrate was then evaporated to dryness under reduced pressure.

Chemical Separations

Liquid-Liquid Partition Chromatography

The crude methanolic extract was resuspended in aqueous methanol ($MeOH_{aq}$) ($MeOH:H_2O$, 3:2). This was partitioned sucessively in order of increasing polarity with petroleum ether (pet. ether) and dichloromethane (CH_2Cl_2).

Thin Layer Chromatography

Silica gel G60 F_{254} alumina backed plates were used with the appropriate solvent system. UV active compounds were detected at 254 and 366 nm, after which the chromatogram was stained with vanillin:H_2SO_4 spray reagent.

Vacuum Liquid Chromatography

TLC grade silica (Merck Kiesel gel 60) was used as the stationary phase, following a procedure modified from Pelletier et al. (1986). The solvent system used was petroleum ether with increasing amounts (0, 1, 2, 5, 10, 25, 50%) of dichloromethane, then dichloromethane with increasing amounts (0, 1, 2, 5, 10, 25%) of methanol. The column was finally washed with 50% MeOH in CH_2Cl_2, then 100% MeOH.

Preparative High Performance Liquid Chromatography (HPLC)

Active fractions were further separated on HPLC using a Waters 660E controller, and the Waters 994 programmable photodiode array (PDA) detector, with either a Waters RCm 8 x 10, or 25 x 10, NV C_{18} column. The UV detector was set to scan for absorption at 300 nm. The solvent condition used was a

gradient of 35:65 MeOH:TFA (0.05% in H_2O) to 65:35 MeOH:TFA (0.05% in H_2O) in 30 min., with a flow rate of 3 mL/min. (for 8 x 10 column) or 20 mL/min. (for 25 x 10 column).

Analytical High Performance Liquid Chromatography (HPLC)

Chromatograms of fractions were obtained by HPLC using the Waters Millenium system, with a Waters 717plus autosampler, Waters 600 controllers, and PDA detector. The column used was a Nova-Pak C_{18} 4 micron, 3.9 x 150 mm HPLC cartridge column. The UV detector was set to scan for absorption at 300 nm. The solvent conditions used were the same as for preparative HPLC, with a flow rate of 1 mL/min..

Structural Analysis

Ultraviolet Spectroscopy (UV-Vis)

Spectra from HPLC fractions were obtained during separation, on the Waters 994 PDA detector.

Mass Spectroscopy (MS)

EI obtained on a Kratos MS 50 mass spectrometer at 70 eV.

DCI was obtained on a Delsi Nermag R10-10 C mass spectrometer with NH_3 as the CI gas. All mass spectrometry was performed by the Mass Spectrometry Center at the Chemistry Department, University of British Columbia.

Nuclear Magnetic Resonance Spectroscopy (NMR)

^{1}H and ^{13}C NMR spectra were recorded in CD_3OD unless otherwise stated, on a Bruker AMX-500 (500 MHz) instrument. The chemical shifts were recorded in parts per million (ppm) on a δ scale calibrated to MeOH (3.30) as an internal standard.

Results

Bioactivity Guided Fractionation

Liquid-Liquid Partitioning

A petroleum ether extract of the aqueous methanol extract (9.6 g), (active at a concentration of 12 μg/mL) was inactive against HSV. A diethyl ether (Et_2O) extract of the aqueous methanol layer was also not active. A $CHCl_3$ extract, however, was active at 25 μg/mL, although the remaining $MeOH_{aq}$ layer was active at 12 μg/mL.

This $MeOH_{aq}$ fraction was subsequently partitioned with petroleum ether, Et_2O, and $CHCl_3$. The petroleum ether layer was partially active against HSV at 6 μg/mL, the Et_2O layer was active at 25 μg/mL, and the $CHCl_3$ extract was the most active at 6 μg/mL).

Fractionation Procedures

The active $CHCl_3$ fraction (2.5 g) was chromatographed on a VLC column (125 g) using petroleum ether with increasing percentage of CH_2Cl_2, (0, 1, 2, 5, 10, 25, 50, 100%), then CH_2Cl_2, with increasing percentage of MeOH, (0, 1, 2, 5, 10, 25, 50, 100%). The resulting fractions (16 fractions at

100 mL each) were collected and tested. The active fraction (3 μg/mL) was eluted with 50% MeOH in CH_2Cl_2 (fraction 14). This fraction (805 mg) was then run on the preparative HPLC.

HPLC chromatography (Waters RCm NV C18 column) was with a solvent gradient of 35:65 MeOH:TFA (0.05% in H_2O) to 65:35 MeOH:TFA (0.05% in H_2O) in 30 min. and an elution rate of 20 mL/min. Three fractions were collected, **1** (0.00-12 min., active at 6 μg/mL), **2** (12-14 min., active at 900 ng/mL) and **3** (14-30 min., active at 400 ng/mL). The chromatogram can be seen in Figure 6.1.

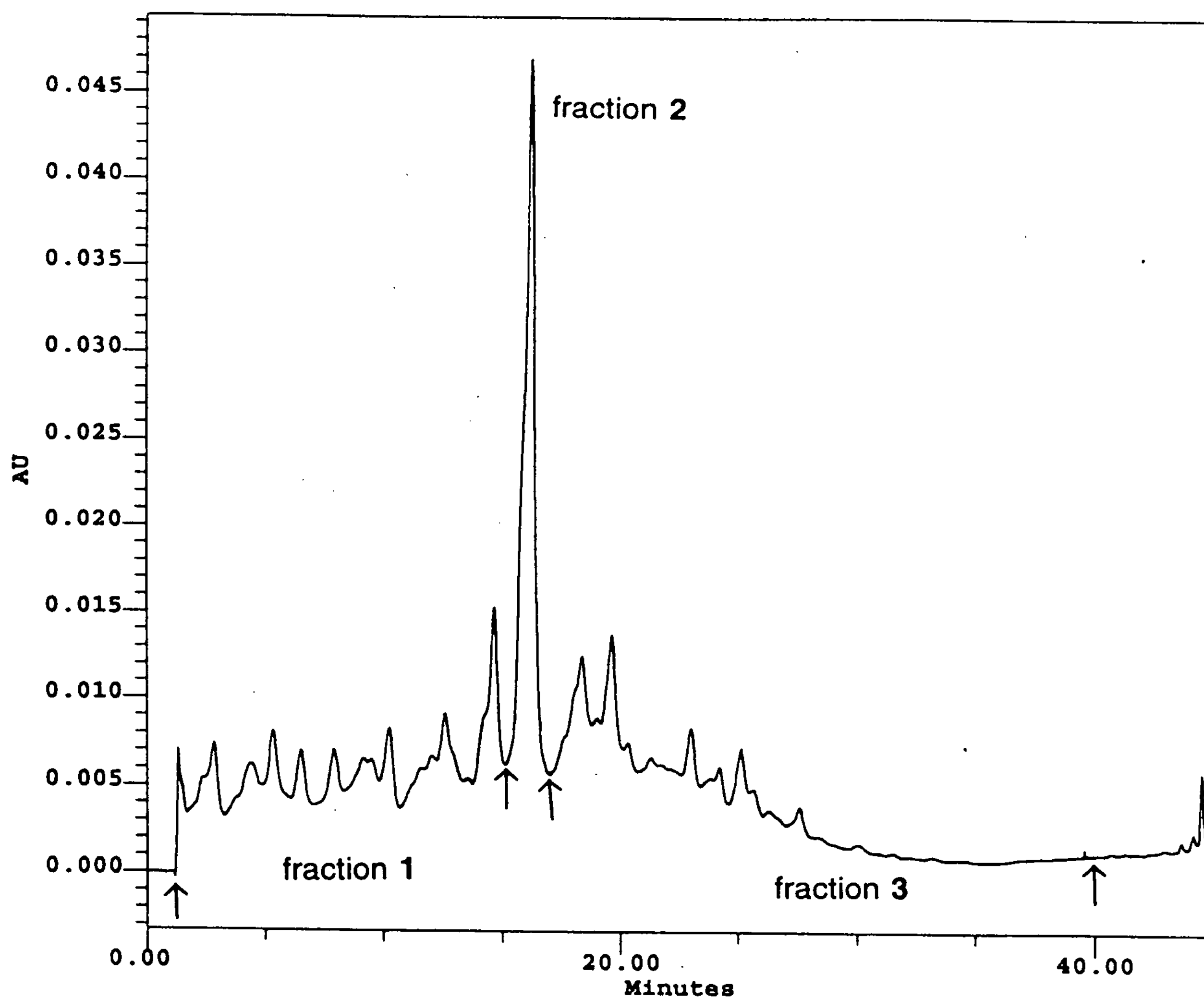


Figure 6.1. HPLC Chromatogram of the active fraction

Chromatogram from analytical HPLC.
(Retention times are different from those of the preparative machine.)

Structural Identification

Fraction **2** was selected for further separation because it contained the major component of the mixture. Fraction **2** was run through the HPLC numerous times until no further purification was achieved. Four milligrams of the pure fraction provided material for structural elucidation.

Fraction **2** was determined to be a mixture by NMR. The UV spectrum of the mixture can be seen in Figure 6.2. The EI spectrum of a solid probe sample with the relative abundance of fragments is shown in Figure 6.3, and the DCI spectrum can be seen in Figure 6.4. The ^{1}H-NMR spectrum can be seen in Figure 6.5. The assignments can be seen in Table 6.1. A ^{13}C-NMR spectrum could not be obtained because there was insufficient material. ^{13}C assignments, listed in Table 6.2, were obtained from HMQC and HMBC experiments. The proton carbon coupling from the HMQC experiment can be seen in Table 6.3, with HMBC results listed in Table 6.4.

One fragment of one component of the mixture, compound **4**, was identified as a derivative of 3,4,5-trimethoxycinnamic acid (Figure 6.6). The methyl ester was prepared by refluxing 3,4,5-trimethoxycinnamic acid (Aldrich, 131 mg) in dry MeOH with a few drops of concentrated H_2SO_4. The resulting product was extracted into Et_2O and the purity was checked by TLC and proton NMR. The antiviral activity of 3,4,5-trimethoxycinnamic acid and the methyl ester of 3,4,5-trimethoxycinnamic acid was assessed against HSV. The methyl ester of 3,4,5-trimethoxycinnamic acid was determined to be partially active against HSV at a concentration of 25 μg/mL, while 3,4,5-trimethoxycinnamic acid was active at 200 μg/mL.

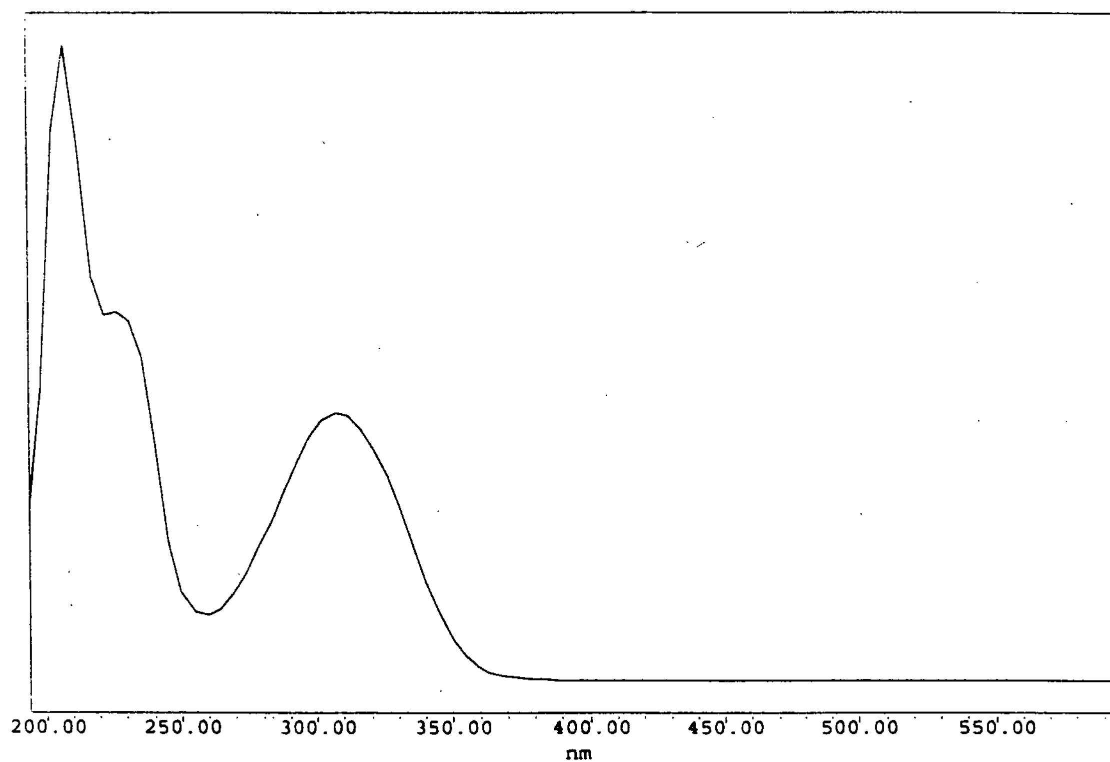


Figure 6.2. UV spectrum of the active fraction of *Carissa carandas*

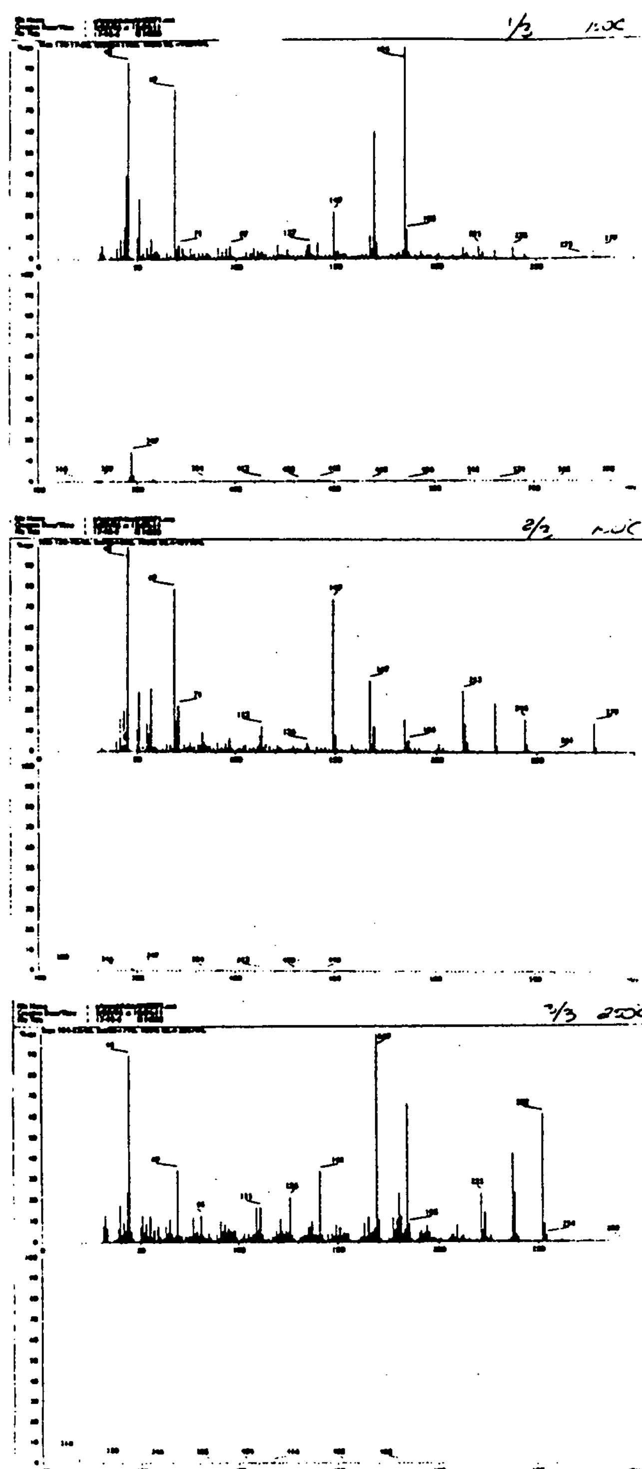


Figure 6.3. EI mass spectra of the active fraction of *Carissa carandas*

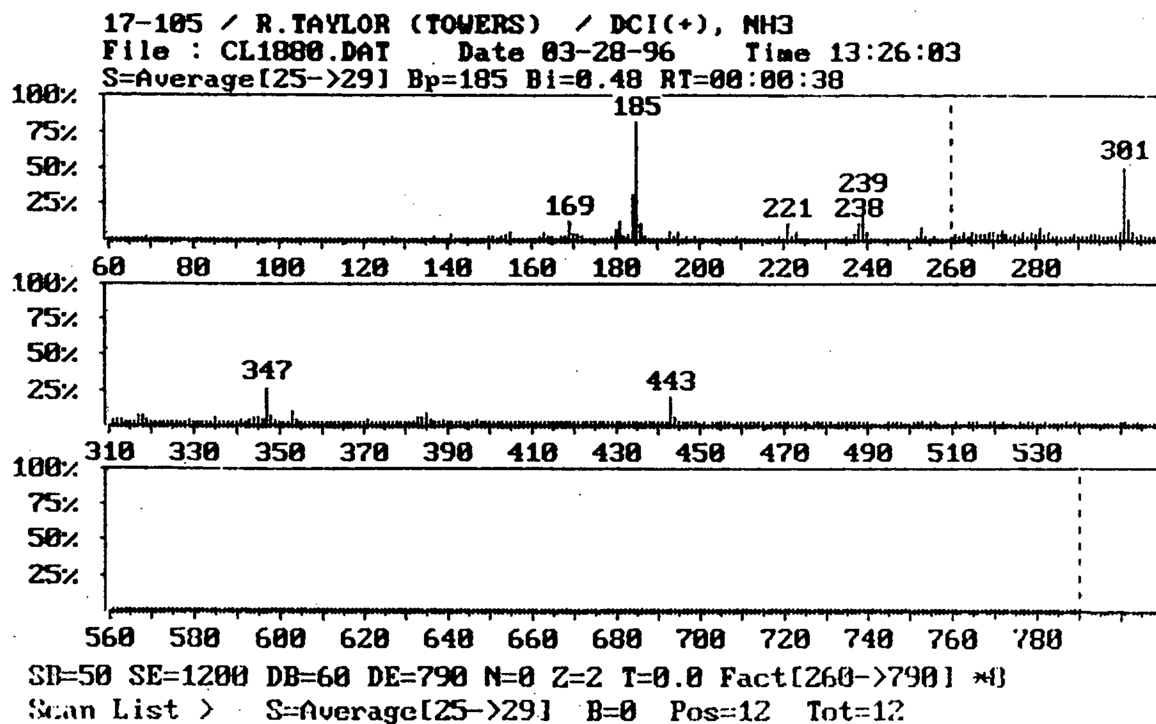


Figure 6.4. DCI mass spectrum of the active fraction of *Carissa carandas*

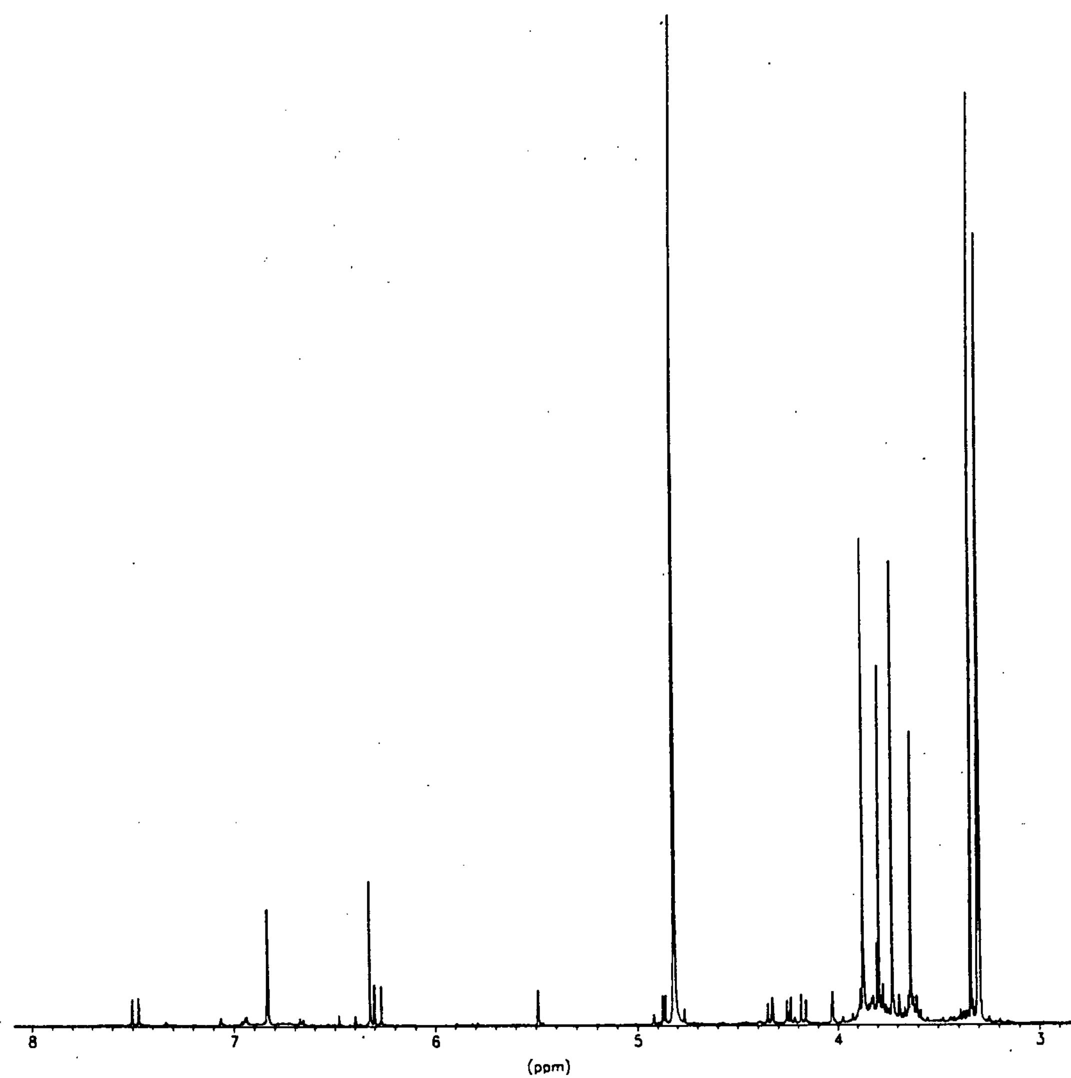


Figure 6.5. ^{1}H-NMR spectrum of the active fraction of *Carissa carandas*

Table 6.1

^{1}H-NMR assignments for the active fraction of *Carissa carandas*

Chemical Shift (ppm)	Compound
7.50 (1H, d, J=16.02)	**4**, proton β to carbonyl
6.80 (2H, s)	**4**, protons on ring carbons 2, 6
6.30 (2H, s)	
6.25 (1H, d, J=16.03)	**4**, proton α to carbonyl
5.45 (1H, d, J=1.15)	
4.35 (1H, d, J=11.25)	
4.25 (1H, d, J=9.73)	
4.20 (1H, d, J=11.44)	
4.02 (1H, s, J=1.15)	
3.87 (6H, s)	**4**, methoxy protons on ring carbons 3, 5
3.79 (3H, s)	**4**, methoxy protons on ring carbons 4
3.72 (6H, s)	
3.63 (3H, s)	
3.35 (3H, s)	

Table 6.2

^{13}C-NMR assignments for the active fraction of *Carissa carandas*

Chemical shift (ppm)	Compound
168.1	**4**, carbonyl of side chain
155.2	
154.6	**4**, C 3, 5 of ring
148.7	
146.7	**4**, C1 of sidechain
141.4	**4**, C4 of ring
134.3	
117.5	**4**, C2 of sidechain
116.0	
110.4	
107.0	**4**, C 2, 6 of ring
103.6	
101.0	
95.5	
94.1	
79.4	
78.48	
75.5	
71.8	
67.3	
62.7	**4**, methoxy at C3, 5 of ring
61.1	**4**, methoxy at C4 of ring
56.5	
55.8	
49.1	
43.7	
35.2	
30.7	
18.9	
17.1	
13.1	

Table 6.3

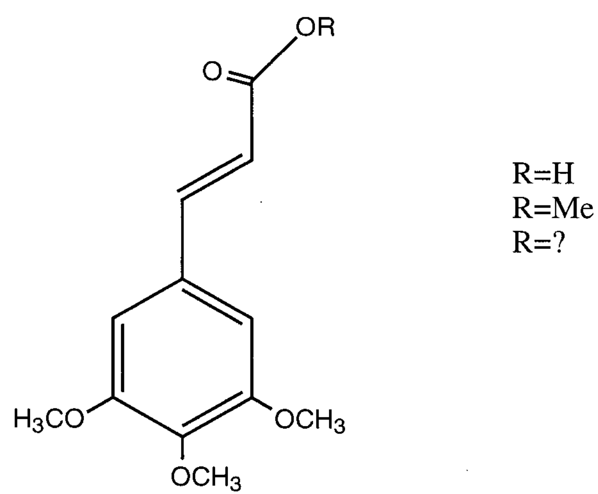
HMQC data for the active fraction of *Carissa carandas*

Chemical shift (ppm)	Proton Correlation
146.7	7.5
117.5	6.3
110.4	5.4
107.0	6.8
103.6	6.8
101.0	4.9
95.5	6.3
79.4	3.6
78.5	4.0, 3.34
75.5	3.9
71.8	3.3
62.7	3.9
61.1	3.8
49.1	3.3

Table 6.4

HMBC data for the active fraction of *Carissa carandas*

Chemical shift (ppm)	Proton Correlation
168.1	7.47
155.2	6.30
154.6	6.8, 3.86, 3.78, 3.71, 3.62 (weak)
148.7	3.78, 3.71
146.7	6.80
141.4	6.8, 3.78
134.3	6.3, 3.62
110.4	3.86
107	7.47, 6.8
103.6	6.8 (weak)
95.5	6.30
94.1	3.78, 3.71 (weak)
78.5	4.35, 4.18, 3.86 (weak), 3.78 (weak)
75.5	5.49
56.5	4.00, 3.71

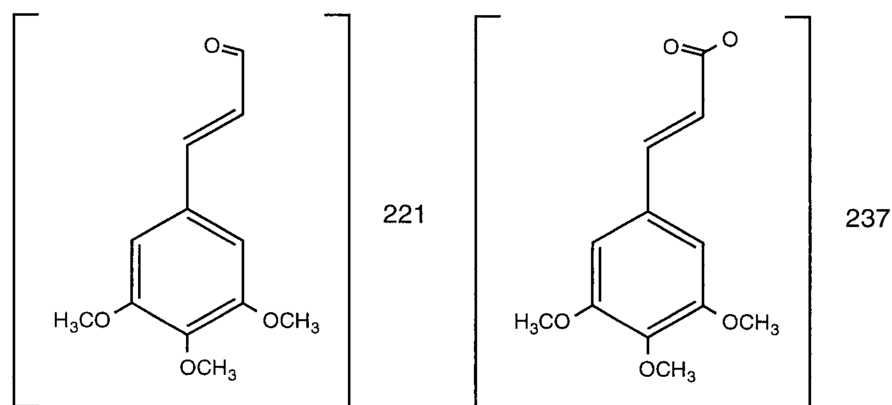


4

Figure 6.6. Proposed Structure of Compound 4

Discussion

Compond **4** was determined to be a component of the active fration **2** based on MS and NMR data. Two peaks observed in the MS at 221 and 237 are characteristic of the following fragments. Another peak in the MS, 252, is characteristic of the molecular ion peak of the methyl ester of 3,4,5-trimethoxycinnamic acid.



From the MS and NMR data, it was determined that the structure of compound **4** contained a 3,4,5-trimethoxycinnamic acid derivative, which remains unidentified (see Figure 6.6).

Five of the peaks on the proton NMR spectrum may be assigned to compound **4**. The methyl protons of the para methoxy group can be seen at 3.79 ppm, with the meta methoxy protons giving a singlet at 3.87. The two ring protons are assigned to the singlet at 6.8 ppm. The signals at 6.15 ppm and 7.5 ppm may be assigned to the α and β protons respectively. The protons have a coupling constant of 16.02 Hz, indicating that the molecule is *trans*. These assignments were made based on the data from the HMQC and HMBC

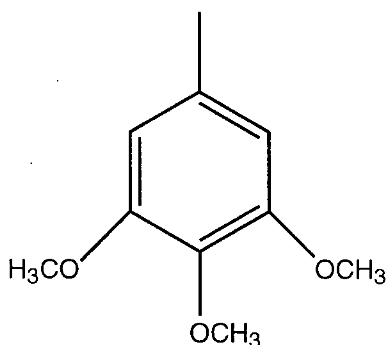
experiments. The proton carbon couplings of the HMBC experiment can be seen in Figure 6.7.

Compound **4** may be responsible for the antiviral activity shown by *C. carandas*. The methyl ester derivative gave moderate activity (25 μg/mL), but did not show the pronounced activity of the mixture (900 ng/mL). This could indicate several possibilities. The methyl ester and/or acid could be present in the mixture, and one or both are responsible, along with another compound, for the antiviral activity (synergistic effects). Another possibility is that compound **4** is a larger structure, containing a 3,4,5-dimethoxycinnamic acid residue. If this compound were responsible for the activity of the mixture, it is not surprising that the acid of methyl ester did not show the same antiviral activity of the mixture. Antiviral activity of caffeoylic acid, 3,4-dimethoxycinnamic acid, and caffeoyl derivatives has been shown by König and Dustmann (1985). These compounds were tested against a variety of animal herpes viruses, including owl, stork and pigeon herpes viruses. Caffeoylic acid was active against all the test viruses, but only to a concentration of 12.5 μg/mL. Serkedjieva and Manolova (1992) tested six esters of substituted cinnamic acids for activity against four strains of influenza virus. All the six compounds showed some activity, with the most potent compound having activity at 25 μg/mL. No methyl esters of trimethoxy substituted cinnamic acids were tested.

As well as phenolics, *C. carandas* is known to contain lignans (Pal et al., 1975), and cardiac glycosides (Vohra and De, 1963; Thorp and Watson, 1953). Both of these classes of phytochemicals are known to have antiviral activity (Hudson, 1990; MacRae and Towers, 1984). It is unlikely that cardiac glycosides are responsible for the antiviral activity seen in *C. carandas* against HSV, as cardiac glycosides which have been tested for antiviral activity have

only moderate activity of approximately 25 μg/mL. Lignans are more likely candidates for the antiviral activity of *C. carandas*. Lignans such as α-(-)-peltatin have antiviral activity at concentrations as low as 10 ng/mL (MacRae et al., 1988). From the MS data, it appears that lignans are too large to account for the activity. α-(-)-Peltatin, for example, has a molecular mass of 400, while 347 and 442 are the largest m/z peaks in the antiviral mixture from *C. carandas*. The 442 peak may be a dimer of 3,4,5-trimethoxycinnamic acid. It is possible that the active component is a lignan like structure containing a subtituted cinnamic acid moiety.

From the NMR data it was also possible to identify another moiety that may be part of compound **4**, or may be a part of the second compound in the mixture. The two equivalent ring protons give a singlet at 6.3 ppm. The *meta* methoxy group protons result in a singlet at 3.72 ppm, while the *para* methoxy protons result in a singlet at 3.63 ppm. This pattern is the same as the compound **4** fragment, but shifted upfeild. This likely indicates that the double bond present in the compound **4** fragment is not present in this second fraction.



The roots of *C. carandas* are known to be poisonous (Joglekar and Gaitonde, 1970), yet the root juice is taken internally to treat dysentery. Joglekar and Gaitonde tested the ethanol extract of the roots of *C. carandas* for histamine liberating activity, and found that it caused an instant rise in blood histamine levels. It also reduced blood pressure in anesthetized cats. It had an LD_{50} of 175 mg/kg in mice, and produced an increase in respiratory rate, drowsiness, vomiting, salivation, defaecation with slimy stools, panting and death in conscious cats. It would be interesting to test the traditional treatment for toxic efffects, and to evaluate it for known toxic substances.

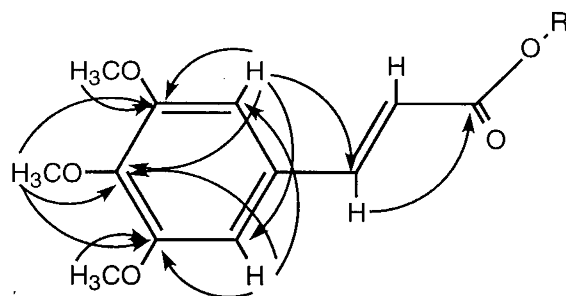


Figure 6.7. HMBC carbon-proton correlations for proposed structure of compound 4

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Chapter VII

Summary Discussion

The scope of this interdisciplinary project was wide, requiring an understanding of aspects of ethnobotany, microbiology, virology and chemistry. It can be summarized as follows.

1. Ethnomedicine in Nepal

   Information about the medicinal uses of various plant species was collected from traditional healers and knowledgeable villagers from a variety of different ethnic groups from the northern, mountainous Langtang and Helambu region, and the south western, flat Terai region of Nepal. Illnesses for which these plants are used are those perceived in western style medicine to be caused by bacterial, fungal or viral pathogens.

2. Plant collection

   Forty-two species were collected and dried in Nepal for eventual antimicrobial screening and chemical analysis.

3. Antimicrobial screening

   These forty-two species were extracted with MeOH and the extracts screened for activity against a variety of bacteria, fungi and viruses, under an assortment of light conditions to test for photosensitizers. Thirty-seven of the forty-two extracts showed activity against bacteria and thirty-five showed activity against fungi. Only eight were active against Gram-negative bacteria. The exposure to UV-A light had a considerable effect on the

activities of some extracts, with eight extracts being active only when exposed to light. The antibacterial and antifungal effect of fifteen extracts were enhanced upon exposure to light. Fifteen of the forty-two extracts showed 100% inactivation of at least one virus, and fifteen showed partial activity. Exposure to UV-A or visible light had a considerable effect on the antiviral activities of some extracts. Eight extracts were active only when exposed to light, and the antiviral effect of eight extracts enhanced upon exposure to light.

4. Isolation and Identification of biologically active compounds

A biologically active fraction from *Centipeda minima* contained three sesquiterpene lactones, identified as 6-O-methylacrylylplenolin, 6-O-isobutyroylplenolin, and 6-O-angeloylplenolin. 6-O-Methylacrylylplenolin had not been previously isolated from *C. minima*. All three of these sesquiterpene lactones had activity against *Bacillus subtilis* and *Staphylococcus aureus*. A fraction from the methanol extract of *Carissa carandas* was quite active against HSV. This fraction was found to contain a derivative of 3,4,5-trimethoxycinnamic acid.

Each part of this study brought up areas worthy of further investigation. During the ethnomedicinal study, for example, a more thorough knowledge of Nepali would have been useful to determine the exact illness a plant was being used to treat. In this same vein, time spent in a clinic with a bilingual doctor would have been useful to learn about the symptoms and vocabulary used to describe certain illnesses encountered in the field. It would also have been interesting not to have limited the ethnomedicine to 'illnesses' caused by viruses, bacteria and fungi. A record of plant use, medicinal or not, would

provide information that could be used by the Nepali government and non-governmental organizations alike when planning land use, sustainable development, reforestation and rural education. Time would not allow for these other ethnobotanical studies.

A change I would make if conducting a study of this type in the future, would be to record how many times a treatment is described by different people, rather than insisting that it be mentioned by a certain number of people before I would record it. This previous method has been used by Manandhar in the past, and he feels that it provides a means to assure the reliability of the information (Manandhar, 1995c). Leaman et al., (1995) in a recent article in the Journal of Ethnopharmacology, use the number of times they are told of a certain remedy as a means to assign it an importance value (IV). This value also describes how many times the treatment was mentioned, and takes into account whether it was in one or more villages. This value can then be correlated to the *in vitro* biological activity of the herbal treatment. Leaman et al. studied malaria remedies, and found that those which had been assigned an IV of four (the highest) were considerably more active than those with an IV of zero.

The screens for biological activity took up a major portion of the time spent on this project. If more time were available, studies on mode of action and possible synergistic effects could prove to be interesting. Especially interesting would be an assay of a series of substituted cinnamic acid esters for anti HSV activity, to determine structure activity relationships. Investigation and structural determination of the other antiviral compounds present in *Carissa carandas* may yield more interesting antiviral compounds.

The ethnobotanical knowledge presented chapter II has been passed down from generation to generation of Nepali, but does not exist in a written

form accessible to the Nepali people. The traditional knowledge obtained in the course of this study has been retranslated into Nepali by N. P. Manandhar (see Appendix 2). This information will be distributed in villages in rural Nepal, and to the Nepalese govenment. The traditional knowledge plus the *in vitro* antimicrobial results, along with any known chemistry of the plant, such as the presence of toxic compounds, will be translated for distribution among government officials and health care workers. It is hoped that these documents will provide community planners and development agencies with the beginning of an inventory of medicinal plants that can be used to insure practical and sustainable development in the future.

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Appendix 1

High Resolution Mass Spectrometry Data

ATOMIC COMPOSITION REPORT

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File Time : 13:23:51
File Type : Hi-Res Mass Spectrum
File Title : Q153-9-1 EI MS50
Source File : c:\mswin\data\h12614.mss
Source Scan(s) : 51:70

Sort Field : M/z (descending).
Scan Filter : none.

Selected Isotopes:

Symbol	Min	Max	V'cy	Name
C	0	25	4	Carbon-12
H	0	auto	1	Hydrogen-1
O	0	8	2	Oxygen-16

Allowable error = minimum of 100.0 ppm, 100.0 mmu.
Ring/Double Bond limits = [-0.5 : 100.0]

Number of Peaks = 20.
Base Peak = 246.12568, 100% Intensity = 146902.

Mass	%age	Calculated	ppm	mmu	R/DB	Formula
335.18124	0.69	335.18585	13.8	4.6	6.5	$C_{19}H_{27}O_5$
		335.17059	-31.8	-10.6	2.5	$C_{15}H_{27}O_8$
		335.16473	-49.2	-16.5	11.5	$C_{22}H_{23}O_3$
		335.20111	59.3	19.9	10.5	$C_{23}H_{27}O_2$
		335.20697	76.8	25.7	1.5	$C_{16}H_{31}O_7$
		335.14948	-94.8	-31.8	7.5	$C_{18}H_{23}O_6$
334.17824	4.45	334.17801	-0.7	-0.2	7.0	$C_{19}H_{26}O_5$
		334.19327	45.0	15.0	11.0	$C_{23}H_{26}O_2$
		334.16278	-46.3	-15.5	3.0	$C_{15}H_{26}O_8$
		334.19916	62.6	20.9	2.0	$C_{16}H_{30}O_7$
		334.15689	-63.9	-21.3	12.0	$C_{22}H_{22}O_3$
333.16485	0.84	333.17020	16.0	5.3	7.5	$C_{19}H_{25}O_5$
		333.15494	-29.8	-9.9	3.5	$C_{15}H_{25}O_8$
		333.14908	-47.3	-15.8	12.5	$C_{22}H_{21}O_3$
		333.18546	61.8	20.6	11.5	$C_{23}H_{25}O_2$
		333.19131	79.4	26.5	2.5	$C_{16}H_{29}O_7$
		333.13382	-93.1	-31.0	8.5	$C_{18}H_{21}O_6$

ATOMIC COMPOSITION REPORT

Mass	%age	Calculated	ppm	mmu	R/DB	Formula
332.16226	5.00	332.16238	0.4	0.1	8.0	$C_{19}H_{24}O_5$ ←
		332.14713	-45.6	-15.1	4.0	$C_{15}H_{24}O_8$
		332.17764	46.3	15.4	12.0	$C_{23}H_{24}O_2$
		332.14124	-63.3	-21.0	13.0	$C_{22}H_{20}O_3$
		332.18350	64.0	21.2	3.0	$C_{16}H_{28}O_7$
304.16620	1.98	304.16745	4.1	1.2	7.0	$C_{18}H_{24}O_4$
		304.15219	-46.1	-14.0	3.0	$C_{14}H_{24}O_7$
		304.18271	54.3	16.5	11.0	$C_{22}H_{24}O$
		304.14633	-65.3	-19.9	12.0	$C_{21}H_{20}O_2$
		304.18860	73.6	22.4	2.0	$C_{15}H_{28}O_6$
276.17180	0.93	276.17255	2.7	0.7	6.0	$C_{17}H_{24}O_3$
		276.15729	-52.5	-14.5	2.0	$C_{13}H_{24}O_6$
		276.18781	58.0	16.0	10.0	$C_{21}H_{24}$
		276.15143	-73.8	-20.4	11.0	$C_{20}H_{20}O$
		276.19366	79.2	21.9	1.0	$C_{14}H_{28}O_5$
265.13908	2.10	265.14398	18.5	4.9	5.5	$C_{15}H_{21}O_4$
		265.12872	-39.1	-10.4	1.5	$C_{11}H_{21}O_7$
		265.12286	-61.2	-16.2	10.5	$C_{18}H_{17}O_2$
		265.15924	76.0	20.2	9.5	$C_{19}H_{21}O$
		265.16510	98.1	26.0	0.5	$C_{12}H_{25}O_6$
264.13508	14.05	264.13617	4.1	1.1	6.0	$C_{15}H_{20}O_4$
		264.12091	-53.6	-14.2	2.0	$C_{11}H_{20}O_7$
		264.15143	61.9	16.3	10.0	$C_{19}H_{20}O$
		264.11502	-75.9	-20.1	11.0	$C_{18}H_{16}O_2$
		264.15729	84.1	22.2	1.0	$C_{12}H_{24}O_6$
263.12823	18.46	263.12833	0.4	0.1	6.5	$C_{15}H_{19}O_4$
		263.11307	-57.6	-15.2	2.5	$C_{11}H_{19}O_7$
		263.14359	58.4	15.4	10.5	$C_{19}H_{19}O$
		263.10721	-79.9	-21.0	11.5	$C_{18}H_{15}O_2$
		263.14948	80.7	21.2	1.5	$C_{12}H_{23}O_6$

ATOMIC COMPOSITION REPORT

Mass	%age	Calculated	ppm	mmu	R/DB	Formula
247.12985	19.18	247.13342	14.5	3.6	6.5	$C_{15}H_{19}O_3$
		247.11816	-47.3	-11.7	2.5	$C_{11}H_{19}O_6$
		247.11229	-71.1	-17.6	11.5	$C_{18}H_{15}O$
		247.14868	76.2	18.8	10.5	$C_{19}H_{19}$
		247.15456	100.0	24.7	1.5	$C_{12}H_{23}O_5$
246.12568	100.00	246.12560	-0.3	-0.1	7.0	$C_{15}H_{18}O_3$
		246.14085	61.7	15.2	11.0	$C_{19}H_{18}$
		246.11034	-62.3	-15.3	3.0	$C_{11}H_{18}O_6$
		246.14673	85.5	21.0	2.0	$C_{12}H_{22}O_5$
		246.10446	-86.2	-21.2	12.0	$C_{18}H_{14}O$
246.08468	0.29	246.08920	18.4	4.5	8.0	$C_{14}H_{14}O_4$
		246.07396	-43.6	-10.7	4.0	$C_{10}H_{14}O_7$
		246.06808	-67.4	-16.6	13.0	$C_{17}H_{10}O_2$
		246.10446	80.4	19.8	12.0	$C_{18}H_{14}O$
245.11710	2.23	245.11777	2.7	0.7	7.5	$C_{15}H_{17}O_3$
		245.10251	-59.5	-14.6	3.5	$C_{11}H_{17}O_6$
		245.13303	65.0	15.9	11.5	$C_{19}H_{17}$
		245.09663	-83.5	-20.5	12.5	$C_{18}H_{13}O$
		245.13890	88.9	21.8	2.5	$C_{12}H_{21}O_5$
237.14447	0.68	237.14906	19.4	4.6	4.5	$C_{14}H_{21}O_3$
		237.13382	-44.9	-10.7	0.5	$C_{10}H_{21}O_6$
		237.12794	-69.7	-16.5	9.5	$C_{17}H_{17}O$
		237.16432	83.7	19.9	8.5	$C_{18}H_{21}$
236.14100	6.53	236.14125	1.1	0.3	5.0	$C_{14}H_{20}O_3$
		236.12599	-63.6	-15.0	1.0	$C_{10}H_{20}O_6$
		236.15649	65.6	15.5	9.0	$C_{18}H_{20}$
		236.12012	-88.4	-20.9	10.0	$C_{17}H_{16}O$
		236.16237	90.5	21.4	0.0	$C_{11}H_{24}O_5$
235.13322	1.51	235.13342	0.9	0.2	5.5	$C_{14}H_{19}O_3$
		235.11816	-64.0	-15.1	1.5	$C_{10}H_{19}O_6$
		235.14868	65.8	15.5	9.5	$C_{18}H_{19}$

ATOMIC COMPOSITION REPORT

Mass	%age	Calculated	ppm	mmu	R/DB	Formula
233.10972	1.83	233.10251	-30.9	-7.2	2.5	$C_{10}H_{17}O_{6}$
		233.11777	34.5	8.0	6.5	$C_{14}H_{17}O_{3}$
		233.09663	-56.1	-13.1	11.5	$C_{17}H_{13}O$
		233.13303	100.0	23.3	10.5	$C_{18}H_{17}$
232.10536	14.47	232.10994	19.7	4.6	7.0	$C_{14}H_{16}O_{3}$
		232.09470	-45.9	-10.7	3.0	$C_{10}H_{16}O_{6}$
		232.08882	-71.3	-16.5	12.0	$C_{17}H_{12}O$
		232.12520	85.5	19.8	11.0	$C_{18}H_{16}$
231.10187	94.99	231.10213	1.1	0.3	7.5	$C_{14}H_{15}O_{3}$
		231.08687	-64.9	-15.0	3.5	$C_{10}H_{15}O_{6}$
		231.11737	67.1	15.5	11.5	$C_{18}H_{15}$
		231.08099	-90.3	-20.9	12.5	$C_{17}H_{11}O$
		231.12325	92.5	21.4	2.5	$C_{11}H_{19}O_{5}$

***** End of Atomic Composition Report *****

ATOMIC COMPOSITION REPORT

File Name : c:\mswin\data\average.mss
File Date : 6/5/96
File Time : 13:53:33
File Type : Hi-Res Mass Spectrum
File Title : Q153-9-2 EI MS50
Source File : c:\mswin\data\h12615.mss
Source Scan(s) : 26:35

Sort Field : M/z (descending).
Scan Filter : none.

Selected Isotopes:

Symbol	Min	Max	V'cy	Name
C	0	19	4	Carbon-12
H	0	auto	1	Hydrogen-1
O	0	5	2	Oxygen-16

Allowable error = minimum of 100.0 ppm, 100.0 mmu.
Ring/Double Bond limits = [-0.5 : 100.0]

Number of Peaks = 18.
Base Peak = 231.10199, 100% Intensity = 123432.

Mass	%age	Calculated	ppm	mmu	R/DB	Formula
335.18269	1.65	335.18585	9.4	3.2	6.5	$C_{19}H_{27}O_5$
334.17760	7.94	334.17801	1.2	0.4	7.0	$C_{19}H_{26}O_5$
265.13989	3.71	265.14398	15.4	4.1	5.5	$C_{15}H_{21}O_4$
		265.12286	-64.2	-17.0	10.5	$C_{18}H_{17}O_2$
		265.15924	73.0	19.4	9.5	$C_{19}H_{21}O$
264.13575	23.71	264.13617	1.6	0.4	6.0	$C_{15}H_{20}O_4$
		264.15143	59.4	15.7	10.0	$C_{19}H_{20}O$
		264.11502	-78.5	-20.7	11.0	$C_{18}H_{16}O_2$
263.12839	19.61	263.12833	-0.2	-0.1	6.5	$C_{15}H_{19}O_4$
		263.14359	57.7	15.2	10.5	$C_{19}H_{19}O$
		263.10721	-80.5	-21.2	11.5	$C_{18}H_{15}O_2$
262.11230	2.00	262.12051	31.3	8.2	7.0	$C_{15}H_{18}O_4$
		262.09937	-49.3	-12.9	12.0	$C_{18}H_{14}O_2$
		262.13577	89.6	23.5	11.0	$C_{19}H_{18}O$

ATOMIC COMPOSITION REPORT

Mass	%age	Calculated	ppm	mmu	R/DB	Formula
247.12926	18.25	247.13342	16.8	4.2	6.5	$C_{15}H_{19}O_3$
		247.11229	-68.7	-17.0	11.5	$C_{18}H_{15}O$
		247.14868	78.6	19.4	10.5	$C_{19}H_{19}$
246.12530	92.70	246.12560	1.2	0.3	7.0	$C_{15}H_{18}O_3$
		246.14085	63.2	15.6	11.0	$C_{19}H_{18}$
		246.10446	-84.7	-20.8	12.0	$C_{18}H_{14}O$
		246.14673	87.1	21.4	2.0	$C_{12}H_{22}O_5$
245.11694	3.07	245.11777	3.4	0.8	7.5	$C_{15}H_{17}O_3$
		245.13303	65.6	16.1	11.5	$C_{19}H_{17}$
		245.09663	-82.8	-20.3	12.5	$C_{18}H_{13}O$
		245.13890	89.6	22.0	2.5	$C_{12}H_{21}O_5$
244.10949	2.56	244.10994	1.8	0.4	8.0	$C_{15}H_{16}O_3$
		244.12520	64.3	15.7	12.0	$C_{19}H_{16}$
		244.08882	-84.7	-20.7	13.0	$C_{18}H_{12}O$
		244.13107	88.4	21.6	3.0	$C_{12}H_{20}O_5$
237.14312	1.95	237.14906	25.1	5.9	4.5	$C_{14}H_{21}O_3$
		237.12794	-64.0	-15.2	9.5	$C_{17}H_{17}O$
		237.16432	89.4	21.2	8.5	$C_{18}H_{21}$
236.14080	12.89	236.14125	1.9	0.5	5.0	$C_{14}H_{20}O_3$
		236.15649	66.5	15.7	9.0	$C_{18}H_{20}$
		236.12012	-87.6	-20.7	10.0	$C_{17}H_{16}O$
		236.16237	91.3	21.6	0.0	$C_{11}H_{24}O_5$
235.13238	1.93	235.13342	4.4	1.0	5.5	$C_{14}H_{19}O_3$
		235.14868	69.3	16.3	9.5	$C_{18}H_{19}$
		235.11229	-85.4	-20.1	10.5	$C_{17}H_{15}O$
		235.15456	94.3	22.2	0.5	$C_{11}H_{23}O_5$
234.12234	0.92	234.12560	13.9	3.3	6.0	$C_{14}H_{18}O_3$
		234.10446	-76.4	-17.9	11.0	$C_{17}H_{14}O$
		234.14085	79.1	18.5	10.0	$C_{18}H_{18}$

ATOMIC COMPOSITION REPORT

Mass	%age	Calculated	ppm	mmu	R/DB	Formula
232.10515	15.94	232.10994	20.6	4.8	7.0	$C_{14}H_{16}O_3$
		232.08882	-70.3	-16.3	12.0	$C_{17}H_{12}O$
		232.12520	86.4	20.0	11.0	$C_{18}H_{16}$
231.10199	100.00	231.10213	0.6	0.1	7.5	$C_{14}H_{15}O_3$
		231.11737	66.6	15.4	11.5	$C_{18}H_{15}$
		231.08099	-90.9	-21.0	12.5	$C_{17}H_{11}O$
		231.12325	92.0	21.3	2.5	$C_{11}H_{19}O_5$

***** End of Atomic Composition Report *****

ATOMIC COMPOSITION REPORT

File Name : c:\mswin\data\average.mss
File Date : 6/5/96
File Time : 14:44:32
File Type : Hi-Res Mass Spectrum
File Title : Q153-9-3 EI MS50
Source File : c:\mswin\data\h12616.mss
Source Scan(s) : 46:55

Sort Field : M/z (descending).
Scan Filter : Minimum Intensity = 2.00%

Selected Isotopes:

Symbol	Min	Max	V'cy	Name
C	0	25	4	Carbon-12
H	0	auto	1	Hydrogen-1
O	0	6	2	Oxygen-16

Allowable error = minimum of 100.0 ppm, 100.0 mmu.
Ring/Double Bond limits = [-0.5 : 100.0]

Number of Peaks = 25, filtered down to 15.
Base Peak = 247.13280, 100% Intensity = 128059.

Mass	%age	Calculated	ppm	mmu	R/DB	Formula
347.18159	3.96	347.18585	12.3	4.3	7.5	$C_{20}H_{27}O_5$
		347.16473	-48.6	-16.9	12.5	$C_{23}H_{23}O_3$
		347.20111	56.2	19.5	11.5	$C_{24}H_{27}O_2$
		347.14948	-92.5	-32.1	8.5	$C_{19}H_{23}O_6$
346.17775	18.20	346.17801	0.7	0.3	8.0	$C_{20}H_{26}O_5$
		346.19327	44.8	15.5	12.0	$C_{24}H_{26}O_2$
		346.15689	-60.3	-20.9	13.0	$C_{23}H_{22}O_3$
278.15106	5.43	278.15179	2.6	0.7	6.0	$C_{16}H_{22}O_4$
		278.16705	57.5	16.0	10.0	$C_{20}H_{22}O$
		278.13068	-73.3	-20.4	11.0	$C_{19}H_{18}O_2$
		278.17294	78.7	21.9	1.0	$C_{13}H_{26}O_6$
264.13405	9.15	264.13617	8.0	2.1	6.0	$C_{15}H_{20}O_4$
		264.15143	65.8	17.4	10.0	$C_{19}H_{20}O$
		264.11502	-72.0	-19.0	11.0	$C_{18}H_{16}O_2$
		264.15729	88.0	23.2	1.0	$C_{12}H_{24}O_6$

ATOMIC COMPOSITION REPORT

Mass	%age	Calculated	ppm	mmu	R/DB	Formula
249.13939	2.24	249.13382	-22.4	-5.6	1.5	$C_{11}H_{21}O_6$
		249.14906	38.8	9.7	5.5	$C_{15}H_{21}O_3$
		249.12794	-45.9	-11.4	10.5	$C_{18}H_{17}O$
248.13650	17.83	248.14125	19.1	4.8	6.0	$C_{15}H_{20}O_3$
		248.12599	-42.3	-10.5	2.0	$C_{11}H_{20}O_6$
		248.12012	-66.0	-16.4	11.0	$C_{18}H_{16}O$
		248.15649	80.6	20.0	10.0	$C_{19}H_{20}$
247.13280	100.00	247.13342	2.5	0.6	6.5	$C_{15}H_{19}O_3$
		247.11816	-59.2	-14.6	2.5	$C_{11}H_{19}O_6$
		247.14868	64.3	15.9	10.5	$C_{19}H_{19}$
		247.11229	-83.0	-20.5	11.5	$C_{18}H_{15}O$
		247.15456	88.0	21.8	1.5	$C_{12}H_{23}O_5$
246.12549	66.76	246.12560	0.4	0.1	7.0	$C_{15}H_{18}O_3$
		246.11034	-61.6	-15.2	3.0	$C_{11}H_{18}O_6$
		246.14085	62.4	15.4	11.0	$C_{19}H_{18}$
		246.10446	-85.4	-21.0	12.0	$C_{18}H_{14}O$
		246.14673	86.3	21.2	2.0	$C_{12}H_{22}O_5$
245.11718	3.25	245.11777	2.4	0.6	7.5	$C_{15}H_{17}O_3$
		245.10251	-59.9	-14.7	3.5	$C_{11}H_{17}O_6$
		245.13303	64.6	15.8	11.5	$C_{19}H_{17}$
		245.09663	-83.8	-20.5	12.5	$C_{18}H_{13}O$
		245.13890	88.6	21.7	2.5	$C_{12}H_{21}O_5$
236.14015	4.63	236.14125	4.7	1.1	5.0	$C_{14}H_{20}O_3$
		236.12599	-60.0	-14.2	1.0	$C_{10}H_{20}O_6$
		236.15649	69.2	16.3	9.0	$C_{18}H_{20}$
		236.12012	-84.8	-20.0	10.0	$C_{17}H_{16}O$
		236.16237	94.1	22.2	0.0	$C_{11}H_{24}O_5$
233.11180	2.08	233.11777	25.6	6.0	6.5	$C_{14}H_{17}O_3$
		233.10251	-39.9	-9.3	2.5	$C_{10}H_{17}O_6$
		233.09663	-65.1	-15.2	11.5	$C_{17}H_{13}O$
		233.13303	91.1	21.2	10.5	$C_{18}H_{17}$

ATOMIC COMPOSITION REPORT

Mass	%age	Calculated	ppm	mmu	R/DB	Formula
232.10547	10.53	232.10994	19.3	4.5	7.0	$C_{14}H_{16}O_3$
		232.09470	-46.4	-10.8	3.0	$C_{10}H_{16}O_6$
		232.08882	-71.7	-16.6	12.0	$C_{17}H_{12}O$
		232.12520	85.0	19.7	11.0	$C_{18}H_{16}$
231.10195	66.59	231.10213	0.8	0.2	7.5	$C_{14}H_{15}O_3$
		231.08687	-65.3	-15.1	3.5	$C_{10}H_{15}O_6$
		231.11737	66.7	15.4	11.5	$C_{18}H_{15}$
		231.08099	-90.7	-21.0	12.5	$C_{17}H_{11}O$
		231.12325	92.1	21.3	2.5	$C_{11}H_{19}O_5$
230.12617	2.04	230.13068	19.6	4.5	7.0	$C_{15}H_{18}O_2$
		230.11542	-46.7	-10.8	3.0	$C_{11}H_{18}O_5$
		230.10956	-72.2	-16.6	12.0	$C_{18}H_{14}$

***** End of Atomic Composition Report *****

Appendix 2
Nepali Translation of Ethnobotanical Information

Some Plants used as medicines
by people of the
Langtang and Helambu regions
of Nepal.

नेपाल को लाँगटाँग तथा हेलम्बु क्षेत्रहरूका
मानिसहरूले प्रयोग गर्ने औषधोपयोगी
बिरुवाहरू

नेपाली नाउँ अँधिजालो
तामाङ नाउँ अधिजाल
यो स्तहरा गरी चिस्यान र ओसिलो ठाउँमा उम्रने झार हो। यसको फूल सेतो हुन्छ। तथा यसको निको पार्न शेर्पा तथा तामाङ समुदायहरूले यो बिरूवाको रस ४ चौथा चम्चा दिनको ३ पटक सम्म सेवन गर्ने गर्दछन्।

तामाङ नाउँ नच्यु म्हेदो
खुला तथा पथरीलो ठाउँ पाखामा पाइने यो एक प्रकारको झुस्यान हो। यसको फूल पहेंलो हुन्छ। शेर्पा र तामाङ दिउँ मलको जराको रस ज्वरो निको पार्न सेवन गर्दछन्।

नेपाली नाउँ: पहेंली
चिस्यान र छायाँ लाग्ने ठाउँमा उम्रने यो बिरूवाको फुल पहेंलो हुन्छ।
शेर्पा तथा तामाङ जातीले यो बिरूवाको ३ चौथा चम्चा रस दिनको ३ पटक सम्म टाइफाइड निको पार्न प्रयोग गर्दछन्।

~~नेपाली नाउँ: वन सिलम~~
~~खुला जग्गामा उम्रने यो एक किसीमको झार हो। यसमा पहेंको रंगको फूल लाग्दछ। यो बिरूवाको ४ चौथा चम्चा रस दिनको ४ पटक सेवन गरेर झाडा पखाला निको पार्ने चलन शेर्पा र तामाङ समुदायमा छ।~~

नेपाली नाउँ: वन सिलाम
पहाडको खुला तथा सोपिलो ठाउँमा उम्रने यो बिरूवाको फूल पहेंलो हुन्छ।
सुकेको जराको ४ ग्राम धुलो पानीसँग दिनको ३ पटक, ज्वरो निको पार्न प्रयोग गर्ने चलन शेर्पा र तामाङ समुदायमा छ।

औषधोपयोगी बिरुवाहरु

नेपाली नाउं: पिनासे झार
तामाङ नाउं: मासिनो म्रो
खुला तथा नदुवाने ठाउँमा पाइने यो एक वानस्पतिक झार हो। यसको फूल फिक्का प्याजी रंगको हुन्छ।
शेर्पा तथा तामाङ समुदायका मानिस हरुले रुघा खोकी लागेमा यो बिरुवा लाई पानीमा केही बेर पकाएर सो को रस २ चोया चम्चा को दरले दैनिक २ पटक गरी १ हप्ता सम्म सेवन गर्दछन्।

तामाङ नाउं: ऊहा खिलम
सेतो फूल फुल्ने यो एक किसीम को वुट्यान हो। यो खास गरी खुला डाँडा पाखा हरुमा पाइन्छ।
तामाङ हरु ले रुघा खोकी निको पार्न बिरुवा को रस सेवन गर्दछन् भने कतै कतै हरियो पात त्यसै पनि खाने गर्दछन्। बिउ (बियाँ) को लेदो बनाई लुतो निको पार्न लगाउँदछन्

तामाङ नाउं: स्याँगार
खुला डाँडा पाखामा हुने यो किसीम को वुट्यान हो। यसमा सेतो रंगको फूल फुल्दछ।
शेर्पा र तामाङ समुदाय हरु ले रुघा खोकी निको पार्न उकीमा जम्या सम्म यो बिरुवा को रस दिनको ८ पटक ४-६ दिन सम्म सेवन गर्दछन्।

तामाङ नाउं: जँवँ लोङदु
यो कहर जातिको बिरुवा हो र प्रायः चिसो चिसो तथा ओसिलो ठाउँमा पाइन्छ।
यस कहरा लाई पानी संग पिंधेर लेदो बनाई जँव खटिरा निको पार्न लगाउँदछन्।

नेपाली नाउं: मूलापाती
तामाङ नाउं: घाजार
यो झार साधारणतया ओसेलो र खुल्ला ठाउँमा उम्रने एक प्रकारको झार हो। यसको फिक्का गुलाफी रंग को फूल फुल्दछ।
यो बिरुवा को जरा लाई पिंधेर लेदो बनाई ~~[illegible]~~ हड्डी निको पार्न लगाउँछन्।

नेपाली नाउँ : सानो सिलग
तामाँग नाउँ : मुरजा
खुला जग्गामा हुने यस कुन्यान को फूल पहेंलो हुन्छ।
यसको सुकेको जराको ५ ग्राम धुलो पानी सँग मिसाएर तीनै पानी खाने चलन शेर्पा र तामाँग समुदाय मा छ।

नेपाली नाउँ : पहरा को कान
चिस्यान र मसिनो माटो भएको ठाउँहरु मा उम्रने यस बिरुवा को फूलको प्याजी रंग हुन्छ।
२ चौथाइ चम्चा यस बिरुवा को रस दिनको ४ पटक खोकी लागेमा शेर्पा र तामाँग समुदाय ले प्रयोग गर्ने चलन छ।

नेपाली नाउँ : गोगटी
तामाँग नाउँ : ओगटी
सेपिलो पहाडको पथरीलो ठाउँमा उम्रने यस कुन्यानको फूल पहेंलो रंग को हुन्छ।
यस बिरुवा को जोका पानी मा उमालो उक्त पानी ले गरर गरी कुल्ला गरेमा घाँटीको इन्फेक्सन र आँप चाटी रोग निको हुन्छ भन्ने शेर्पा र तामाँग समुदाय मा छ।

नेपाली नाउँ : खपर
तामाँग नाउँ : खयार
खोंड भएको कुन्यान वा सानो रूख हुने यो बिरुवा धेरै जसो खुला र पथरीलो जग्गामा उम्रेको पाइन्छ। यसमा फिक्का पहेंलो रंगको फूल लाग्दछ।
यसको जरा पोटेर पाइ गरेको मा लगाउने चलन तामाँग समुदाय मा छ।

नेपाली नाउँ : हाडच्यु झार
तामाँग नाउँ : हाडि म्रो
सनचर परेको खुला जग्गामा उम्रने यो सानो खालको बिरुवाको फूलहरु फिक्का हरियो हुन्छन्।
तामाँग समुदायमा हड्डी खोकी लागेको बेला यस बिरुवा को फूल को उपलब्ध परेको गाउँ मा धुलो सुकाएर वा उमालेर पानी मा मिसाएर एक प्याला दिन खाने चलन छ।

तामाङ नाउँ : नचु
यो एक किसीमको सानो बिरुवा हो र यसको फुल पहेंलो हुन्छ।
जरो आएको बेला यस बिरुवाको जराको रस पिउने प्रचलन शेर्पा र तामाङ समुदायमा छ।

नेपाली नाउँ : कुम कुम
छायाँ परेको र गलिलो पार पात भएको डुंगाहरुमा हुने यो एक किसिमको सानो बिरुवा हो। यसको फुल पहेंलो हुन्छ।
रुघा खोकी निको पार्न शेर्पा समुदायमा यस बिरुवाको छ थोपा चम्चा रस दिनको २ पटक खाने चलन छ।

नेपाली नाउँ : रतनजोत
तामाङ नाउँ : रिंफाबे
खुला र पथरिलो जग्गामा उम्रीने यस बिरुवाको फुलको रंग सेतो हुन्छ।
यस बिरुवाको जराको रस २ थोपा चम्चा दिनको ३ चोटी, रुघा खोकी निको पार्न शेर्पा हरुले प्रयोग गर्दछन्।

तामाङ नाउँ : नमलो जगानसी
छायाँ परेको र ओसिलो भू भागमा उम्रीने यस बिरुवाको फुलको रंग सेतो हुन्छ।
शेर्पा समुदायमा आँखा सम्बन्धि रोग निको पार्न यस बिरुवाको जराको रस आँखामा राख्ने दुखीकोर निको पार्न प्रयोग गर्दछन्।

नेपाली नाउँ : मलाटो
तामाङ नाउँ : काला
समशितोष्ण जंगलमा पाइने यस रुखको फुल पहेंलो हुन्छ।
शेर्पा र तामाङ समुदायमा यस बिरुवाको जराको रस जरो निको पार्न प्रयोग गर्दछन्।